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TESIS DOCTORAL

Expression and functional analysis of the Notch signaling pathway within the thymus microenvironment

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A mis padres,

“There is no energy in matter other than that received from the environment”

“No hay mas energía en la materia que aquella recibida del entorno”.

–Nikola Tesla–

Preface

On the cover: *Logos, Eros* and *Thumos.*

Plato famously divided the human *Psyche* into three parts by using the allegory of a charioteer driving two winged horses. The charioteer is *Logos* (the Reason), the black horse is *Eros* (the Desires) and the white horse is *Thumos* (the Emotions). Thus, the task of *Logos* is to balance human desires and emotions, as both must be guided by rationality (1, 5, 6). Eros is a dark wild horse, hedonist and stubborn. He constitutes our appetites at our lowest level, embracing the desire to eat, sleep, reproduce, and live just for pleasure. Conversely, *Thumos* (also commonly spelled as "Thymos"; θυμός: anger) is bright and beautiful, governable, ruled by the command of *Logos*, and helps the reasonable Charioteer to control the passionate dark horse (4). *Thumos* is known to us from the herb thyme, which exudes a strong aroma. "Thyme" is the cognate word of "Fume" in latin (as Th and f is the same Indo-European consonant) thus its original meaning is smoke, "breath", exhalation, "Spirit" or "soul". In the Homeric epics the Greek philosopher Empedocles often used *Thumos* as the vital principle, the "breath of life", "the site for the soul" (3, 4). *Thumos* is also known by the primary lymphoid organ, named as "thymus" by Galen (Pergamum, c.130 A.D.) due to its histologic resemblance with the thyme herb (Fig. I) (3) and its anatomic location at the upper chest where strong emotions are physically felt (no, it's not at the heart, it's at the thymus) (4). As being the site for the soul the thymus involutes with age along with our strengths, passions and wishes.

Thumos takes several emotions as pride, indignation, competitiveness and ambition, although its fundamental manifestation is anger, but of a special kind. Anger is not a pure emotion as it is usually accompanied by and idea, some conviction about our rights, our privileges, or our status (6). Thus, *Thumos* arises when your honor or reputation is questioned or when your efforts, rights or qualities are not recognized. *Thumos* constitutes the fundamental human need for recognition. Lack of recognition turns into anger and drives a person to stand up for itself. *Thumos* is, in modern psychology, our self-stem or self-worth, and thus it names several mood disorders as dysthymia, athymia or cyclothymia (4). *Thumos* also can make you angry with yourself when you fail to live up to your own principles and code of honor (dictated by *Logos*, the Reason) and make you indignant of your own desires, if those oblige you to do something contrary to the dictates of Reason. In contrast to the lower desires of the dark horse, moved for pleasure, *Thumos* seeks independence over wealth, and recognition and honor over security. This drive for recognition will motivate him to risk much, for his reputation an also for the reputation of a group to which he is devoted (4, 6).

Thumos pushes you to despise mediocrity and to want to excel your fellows, to dominate, and to seek glory and legacy. *Thumos* is courage and self-confidence, and it will keep you in a fight that your Reason (Logos) has decided is indeed a worthy one (1). Great primates know that their courage and confidence comes from their *Thumos*, as before a fight they stimulate it by beating their chest above the heart. But just like the dark horse of our appetites, the White Horse of *Thumos* can be harmful, as being ruled by emotions is dangerous if they usurp the role of Reason, the Charioteer. Conversely, extreme retention of your white horse by the Reason will make you susceptible to external insults and to be ruled by the desires and emotions of others (4). Intriguinly, the thyme oil obtained from the thyme herb helps to encourage a drained white horse against external insults as it is an effective antiseptic (7) and antinflamatory (8).

Altogether, the whole ancient knowledge about the *Thumos*, draw a picture of an holistic force that protects and encourages human individuality. *Thumos* is the force that arises to vindicate ourselves and draw a line, based in our unique experiences and dictates of the Reason, that external insults must not cross. Indeed, the cells generated in the thymus can be found from our nose tip to our toes, and are always ready to fight against external threats, thus perfectly materializing the global and individually unique protective force described by the ancient Greeks.

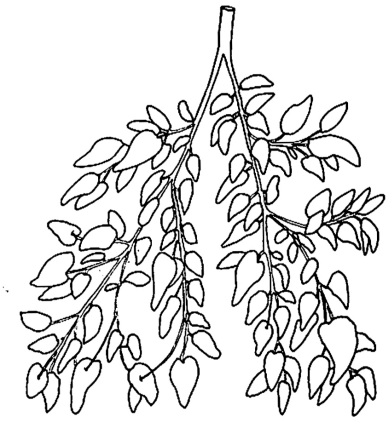

Figure I. Architectural resemblance of thyme herb branches with the polylobulated histology of the thymus gland (3).

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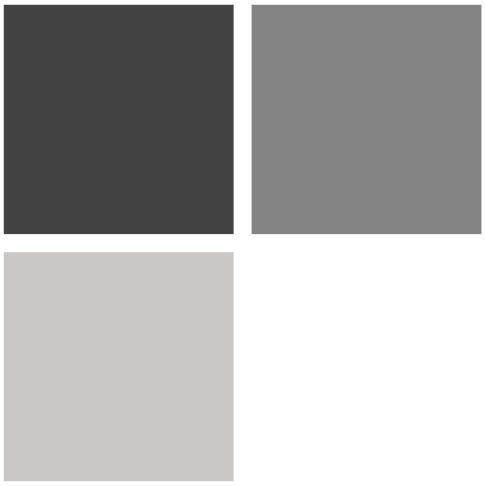

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List of abbreviations

AGM: Aorta-Gonad-Mesonephros.

APC: Antigen presenting cell.

BM: Bone marrow.

CB: Cord blood.

CMJ: Corticomedullary junction.

cTEC: Cortical thymic epithelial cell.

Dll1: Delta-like1.

Dll4: Delta-like4.

DN: Double-negative.

DP: Double-positive.

EC: Endothelial cell.

Ed: Embryonic day.

ETP: Early thymic progenitor.

FFPE: Formalin-fixed paraffin-embbed.

FTOC: Fetal thymic organ culture.

GSI: Gamma-secretase inhibitor.

GvDH: Graft versus host disease.

HC: Hassall's corpuscles.

HPC: Hematopoietic progenitor cell.

HSA: Heat shock antigen.

HSC: Hematopoietic stem cell.

IC: Inner cortex.

ICN: Intracelullar Notch.

icTCRβ: Intracelullar TCRb.

IFN: Interferon.

Jag1: Jagged1.

Jag2: Jagged2.

LSK: Lineage, Sca-1, SCF receptor Kit.

MC: Mesenchymal cell.

MFI: Mean fluorescence intensity.

MHC: Major histocompatibility complex.

mTEC: Cortical thymic epithelial cell.

OCT: Optimal cutting temperature.

PB: Peripheral blood.

PMA: Phorbol myristate acetate.

PVS: Perivascular space.

ROI: Region of interest.

SCC: Subcapsular cortex.

scTEC: Subcapsular thymic epithelial cell.

SP: Simple-positive.

T-ALL: T cell acute lymphoblastic leukemia.

TCR: T cell receptor.

TEC: Thymic epithelial cell.

TEPC: Thymic epithelial progenitor cell.

TME: Thymic microenvironment.

TNF: Tumor necrosis factor.

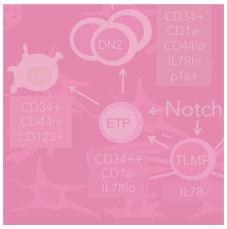
WT: Wild type.

Summary

The thymus provides a specialized environment for T cell development, but the identity of the thymus niche components involved in this process and the nature of their functional interactions with developing thymocytes are still not well understood, specially in humans. The Notch pathway is one of the critical signalling pathways that make the thymus a unique site for T cell development. Notch signalling is delivered by thymic epithelial cells (TECs) expressing distinct Notch ligands to thymus-seeding T cell progenitors expressing several Notch receptors, mainly Notch1. As T-cell development implies the migration of developing thymocytes throughout distinct thymic niches, we ought to determine whether a differential distribution of Notch ligands and receptors in the thymus could define discrete microenvironments with specific developmental functions, paying special attention to a critical check-point that controls the split of developing thymocytes into αβ and γδ T cells. To this end, we performed immunohistochemistry and confocal microscopy analyses focussing on the regulation of the expression of Notch pathway components during human ontogeny in comparison with mouse ontogeny. The study provided information about the localization of human early CD34+ intrathymic progenitors receiving Notch1 signals *in vivo*. In addition, they provided evidence of an spatio-temporal regulation of the expression of Notch ligands and receptors and confirmed that this expression defines particular intrathymic niches, which are dynamically regulated during thymus ontogeny and involution, but differ between mouse and human. Notably, functional *in vitro* assays showed that the opposite pattern of expression observed for Jag1 and Jag2 ligands in the human thymus correlated with divergent functional roles, as they differentially regulate the αβ versus γδ T cell output from human intrathymic progenitors, and also the process of γδ T cell expansion and functional maturation. In fact, Jag1 and Jag2-expressing niches host different γδ maturation stages in the human thymus, thus defining the intrathymic γδ T cell developmental dynamics. Finally, the study provides evidence that Notch signalling may play a key role also in human TEC biology, as not only thymic progenitors, but also TECs express active Notch1 and Hes1. Particularly, the finding that Notch1 activation increases with age in TECs located in the thymus medulla suggests a role for Notch signalling on the natural process of thymic involution. By using a conditional loss of function murine model we show that thymi of mice with impaired Notch activation in the TEC compartment have a disturbed medullary microenvironment, support a defective T-cell development, and allow for aberrant B-cell generation. Altogether the study reveals a crucial role for Notch signalling in a dynamic crosstalk between thymus components, and highlights the importance that specific microenvironments defined by spatio-temporal regulation of Notch ligand expression may have not only in T-cell fate specification, but also in γδ T cell development, TEC biology and thymus involution.

Resumen

El timo proporciona un microambiente especializado para el desarrollo de las células T, pero ni la identidad de los nichos responsables de este proceso, ni la naturaleza de sus interacciones funcionales con los timocitos en desarrollo se conocen bien, especialmente en humanos. La vía de Notch es una de las vías de señalización que hacen del timo un nicho único para la generación de los linfocitos T. Los progenitores hematopoyéticos, que principalmente expresan principalmente el receptor Notch1, reciben señales activadoras de las células epiteliales del timo (TECs) que expresan diversos ligandos. Como el desarrollo de las células T implica la migración de los timocitos a través de distintos nichos intratímicos, hemos querido determinar si una distribución diferencial de receptores y ligandos de Notch en el timo podrían definir microambientes discretos con funciones específicas en el desarrollo T, prestando especial atención al punto de control crítico que determina la bifurcación de los progenitores intratímicos en células de linaje αβ o γδ. Para ello, y mediante inmunohistoquímica y microscopía confocal, hemos analizado la regulación de la expresión de los componentes de la vía Notch durante la ontogenia humana y la de ratón. El estudio ha proporcionado información acerca de la localización *in vivo* de los progenitores CD34+ humanos, los que se activa el receptor Notch1. Además, hemos obtenido evidencia de una regulación espacio-temporal de la expresión de ligandos y receptores Notch, confirmando que Notch define nichos específicos regulados de forma dinámica durante la ontogenia y la involución del timo, que difieren entre ratón y humanos. Adicionalmente, ensayos funcionales *in vitro* han mostrado que el patrón opuesto de expresión de Jag1 y Jag2 observado *in vivo*, correlaciona con funciones divergentes en la regulación de los linajes T γδ y αβ, así como en el proceso de expansión de células T γδ y en su maduración funcional. De hecho, los dos nichos definidos por la expresión de Jag1 y Jag2, albergan diferentes etapas de la maduración de los timocitos γδ, definiendo así la dinámica de diferenciación intratímica de las células T γδ. Por último, nuestro el estudio demuestra que la activación de Notch1 también desempeña un papel clave en la biología del estroma tímico, ya que no sólo los progenitores del timo, sino también las TECs expresan Notch1 activo y Hes1 *in vivo*. En concreto, el hallazgo de que la activación de Notch1 en TECs aumenta con la edad sugiere su implicación en la involución natural del timo. Mediante el uso de un modelo de pérdida de función en ratón, hemos demostrado que los timos de ratones defectivos en la activación de Notch en TECs, presentan alteraciones en el microambiente medular, así como un desarrollo defectuoso de linfocitos T, y una generación aberrante de células B. Por tanto, nuestro estudio revela un papel crucial de Notch en la dinámica de interacciones entre diversos componentes celulares del timo, y pone de relieve la importancia que los nichos específicos definidos por la regulación espacio-temporal de la expresión de los ligandos de Notch pueden desempeñar, no sólo en la especificación del linaje T, sino también en el desarrollo de las células T γδ, en la biología de las TECs y en la involución del timo.



Introduction

1. The thymus: a specialized environment for T cell development.

The environment is the master driver of development in any biological system. In multicellular organisms, the development of any cellular lineage is dependent on the niche that hosts the pluripotent stem cell. In their niche, stem cells receive complex and well-ordered signals that develop their inner potential or allow their self-renewal.

For the hematopoietic system, all hematopoietic linages derive from a common progenitor that resides in primary sites of hematopoiesis such as the yolk sac, AGM and liver in the fetus, and the bone marrow in the adult (Marcos et al., 1997) (Cumano et al., 2000). Most of the hematopoietic lineages mature in those primary sites (Adolfsson et al., 2005) (Luc et al., 2008) (Bell and Bhandoola, 2008) (Graf, 2008), with the exception of T cell lymphocytes (Fig. 1). T cell progenitors need to migrate to an specialized environment for their development, namely the thymus. The thymus provides specific and step-wise signals to developing thymocytes that induce their proliferation, differentiation and migration through different thymic microenvironments (TMEs) (Takahama, 2006) (Petrie and Zuniga-Pflucker, 2007). Little is known about the identities and functional interactions of the thymic niche components *in vivo* with thymic progenitors and developing thymocytes. The characterization and understanding of complex molecular signals that make the thymus the unique niche for T cell development, is the main goal of this PhD thesis.

1.1. Anatomy and development of the human and murine thymus.

The thymus is a primary lymphoid organ of endodermal origin (Manley and Blackburn, 2003) (Gordon et al., 2004) (Hollander et al., 2006) located at the mediastinum and formed by two symmetrical lobules. Comprehension of thymic development will help to understand postnatal thymic anatomy and its specialized niches.

Thymic development can be divided into two phases: pre-vascular and post-vascular development (Boehm, 2008). In the first one, thymic development depends exclusively on epithelial-mesenchymal interactions, while in the later thymic development is dependent also on lympho-stromal interactions (Anderson et al., 1993). Human thymic development starts at the 4th week of gestation (Ed 9.5 - 10.5 in mice), when the thymus is an avascular epithelial rudiment attached to the 3rd pharyngeal pouch, devoid of hematopoietic cells and surrounded by a thick capsule of mesoderm-derived mesenchymal tissue (Fig. 2).

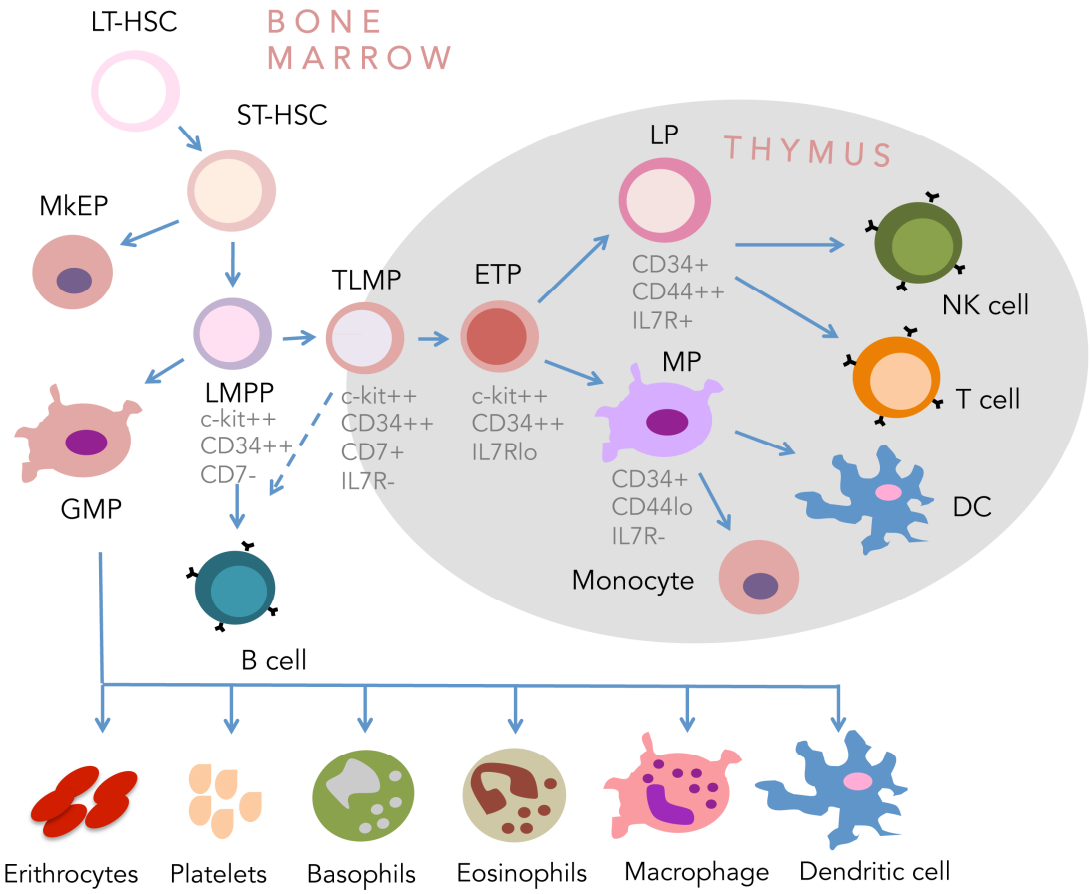


Figure 1. Hematopoietic cell lineages differentiation. BM progenitors give rise to all hematopoietic lineages. T cells are only generated in the thymus. The phenotype of human intrathymic precursor is noted. *LT-HSC*: Long-term self-renewing stem cell; *ST-HSC*: Short-term self-renewing stem cell. *LMPP*: Lympho-myeloid pluripotent progenitor; *MkEP*: Megakariocyte-erythroid progenitor; *GMP*: Granulocyte-monocyte progenitor; *TLMP*: Thymic lymphomyeloid progenitor. *ETP*: Early thymic progenitor; *LP*: Intrathymic lymphoid progenitor; *MP*: Intrathymic myeloid progenitor. Adapted from (Luc et al., 2008).

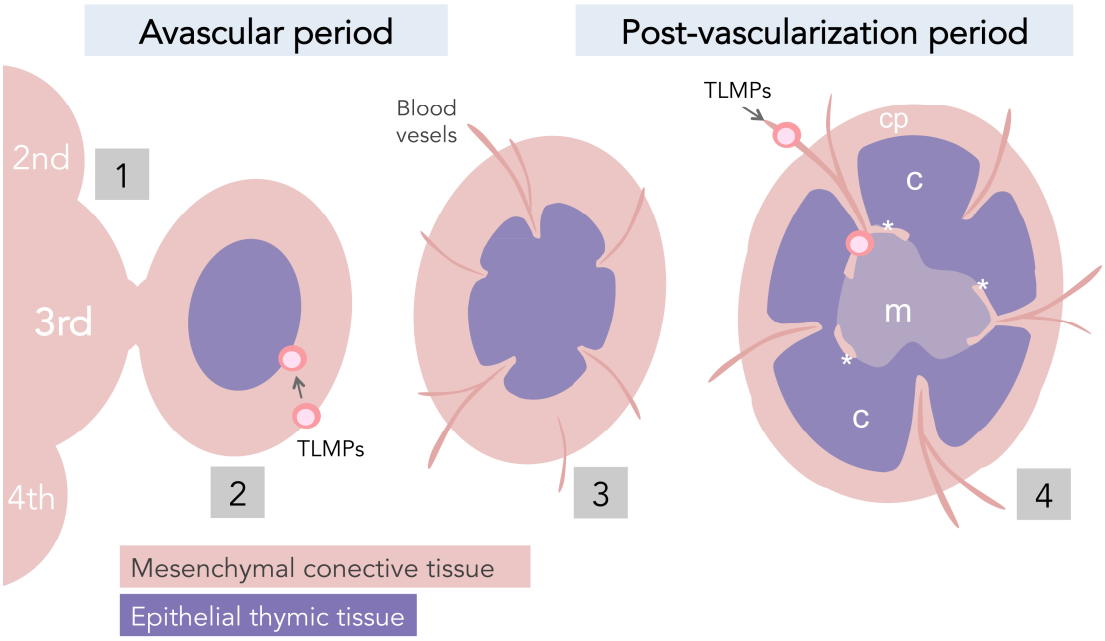


Figure 2. Development of the thymus. Image represents different stages of thymic ontogeny. (1) The thymic anlage develops from the 3rd pharyngeal pouch. (2) Induction of FoxN1 expression and homing of the first wave of TLMPs that enter the thymic anlage by active migration throughout the perithymic mesenchyme. (3) Detachment from the pharinx, vascularization and lobulation. (4) Definitive postnatal anatomy of the thymus; second and successive waves of TLMPs enter thought the blood vessels. *cp:* thymic capsule. *c:* cortex; *m:* medulla; asterisks: perivascular spaces (PVS); TLMP: thymus lymphomyeloid progenitor.

At the beginning of the 8th week of gestation, both left and right thymic rudiments move caudally and fuse, forming the two characteristic thymic lobules (Lobach and Haynes, 1987). The expression of the epithelial transcription factor FoxN1 by thymic epithelial cells (TECs) in late 8th week (Ed11,5 in mice) initiates the process of hematopoietic colonization and the vascularization of the thymic anlage. Indeed, FoxN1-deficient (*nude*) thymic anlages fail to vascularize (Mori et al., 2010). But it is not until the 10th week of gestation (Ed12 in mice), when the mesenchymal connective tissue begins to invaginate into the thymic epithelial anlage along with blood vessels, forming the characteristic discrete lobules of the thymic tissue (fig. 2.3). Additionally, the hematopoietic capacity of the thymus also depends on the activity of the FoxN1 transcription factor (Corbeaux et al., 2010; Nehls et al., 1994). FoxN1 up-regulates the expression of the thymus expressed chemokine (TECK or CCL21) (Liu et al., 2006) and of Notch ligands (Xiao et al., 2008), mostly Dll4 (Tsukamoto et al., 2005). The former attracts the hematopoietic progenitor cells (HPCs) resident in the fetal yolk sac and liver into the thymic anlage (Bleul and Boehm, 2000), and the latter specifies T cell lineage fate on them (Lefort et al., 2006); (Koch et al., 2008) (Hozumi et al., 2008). In FoxN1-deficient animals (*nude*) no hematopoietic cells can be found in the thymus (Blackburn et al., 1996), although their progenitors are produced normally in the bone

marrow (Nehls et al., 1996) (Wortis et al., 1971). Importantly, while hematopoietic colonization of the murine thymus occurs in the 2nd third of gestation (Ed11,5), the human thymus is colonized by HPCs during the 1st third (Fig. 3). As early as the 7th week, CD7+ CD45+ HPCs are found throughout the perithymic mesenchyme and other mesenchymal areas, and by the 8th week the first HPCs colonize the epithelial parenchyma (Lobach and Haynes, 1987) (Haynes et al., 1988). At 8.2 weeks, human thymocytes already express CD8, CD4 and intracellular TCR-β, while in mice, most thymocytes at the equivalent stage (Ed11,5) are CD8- CD4- (DN). By the 10th week, CD3 and CD8β membrane expression is present in fetal thymocytes, and definitive corticomedulary organization of thymocyte populations in cortical immature (CD1a+) and medullary mature (CD1a-) populations is achieved by week 13th. Thus, postnatal histologic configuration of the thymus in cortical and medullary TMEs, is already established in humans by the end of the first trimester (14 wks) (Lobach and Haynes, 1987) (Haynes and Heinly, 1995).

Murine ontogeny	1st week	2nd week	3rd week
	Ed1 – Ed7	Ed8 – Ed14	Ed15 – Ed21
EVENTS:	Thymic fate not specified yet.	Ed9.5: - Thymic anlage formation. Ed11.5: - FoxN1 expression. - Colonization. - Vascularization starts.	Ed15,5: - TCRβ expression. Ed16,5: - Postnatal configuration. - Full T cell repertoire.
Thymocyte phenotype:	Anlage devoid of T cells.	DN	DP CD3+ / SP

Human ontogeny	1st trimester	2nd trimester	3rd trimester
	Wk1 – Wk13	Wk14 – Wk27	Wk28 – Wk40
EVENTS:	· Wk4: - Thymic anlage formation. · Wk8: - FoxN1 expression. - Colonization. - Vascularization starts. ·Wk8.2: - TCRβ expression.	Wk15: - Postnatal configuration. - Full T cell repertoire.	- Postnatal configuration.
Thymocyte phenotype:	DP	DP CD3+ / SP	DP CD3+ / SP

Figure 3. Compared developmental kinetics of thymic development in humans and mice. Table represents human and murine ontogeny divided by thirds. Capital developmental events occurring on each range of time and phenotype of T-lineage cells hosted in the thymus are noted.

Once mature, each thymic lobule is histologically divided into three main compartments: an epithelial cell-rich medulla, surrounded by a lymphocyte-rich cortex, with a perivascular space (PVS) between both. The PVS derives from the early fetal perithymic mesenchymal tissue that penetrates into the thymic parenchyma around the blood vessels during vascularization (Lobach and Haynes, 1987). Additionally, the thymic cortex can be divided in other three functional subregions: subcapsular cortex (SCC), inner cortex (IC) and

corticomedulary junction (CMJ) (Griffith et al., 2009) (Fig. 4). In the postnatal thymus, early T cell progenitors (ETPs) are located at the CMJ (Lind et al., 2001), suggesting that, in the vascularized thymus, ETPs enter through the vasculature and PVS. ETP entrance to the thymus is temporarily regulated in colonization waves, coincident with space availability in limited niches (Donskoy et al., 2003).

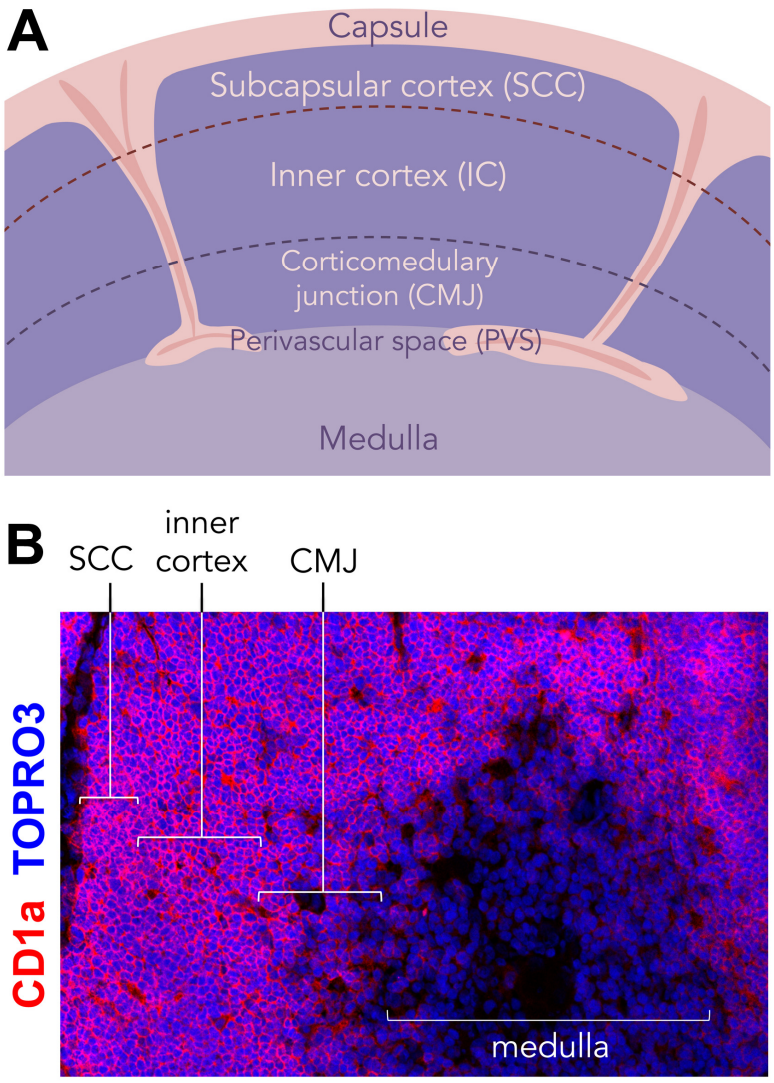


Figure 4. The thymus architecture. *A,* Schematic view of histologic and functional thymic regions. Cortex, medulla and perivascular space are the three formal compartments of the thymus, but the cortex is subdivided in three functional subregions (dashed lines): the subcapsular cortex (SCC), the inner cortex (IC) and the corticomedulary junction (CMJ). *B,* Human postnatal thymus stained for the T cell maturation marker CD1a (red). Thymic regions are limited by brackets.

The functionally mature medullary compartment is histologically defined by the appearance of terminally differentiated medullary TECs (mTECs). These cells are highly keratinized and form rounded swirls known as Hassall's corpuscles (HCs), which are known to be a source of a cytokine known as thymic stroma-derived lymphopoietin (TSLP) (Ziegler and Liu, 2006) that plays a role in dendritic cell (DC) maturation and regulatory T cell (Treg) generation (Watanabe et al., 2005). Additionally, HC's mTECs express the chemokine SDF-1 and the CD30L, a membrane-associated glycoprotein involved in T-cell signaling (Zaitseva et al., 2002) (Romagnani et al., 1998). CD30-deficient mice show a gross defect in negative but not positive selection (Amakawa et al., 1996), suggesting that HCs are capital to negative selection. In the mice, keratinized mTECs form analogous small structures but much more infrequent. This ultimate postnatal thymic structure is not steady, as TECs are in continuous homeostasis during postnatal life (see 3.3).

1.2. The thymic stromal cells.

Early studies in vertebrates demonstrated that the non-lymphoid components of the thymus play critical roles in thymic development and T cell generation (Haynes, 1984). In addition to thymocytes, the thymus contains epithelial cells, fibroblasts, macrophages, dendritic cells (DCs), and vascular endothelial cells, as well as minor populations of myoid cells, B cells, basophils, and eosinophils (Boyd et al., 1993) (Jablonska-Mestanova et al., 2013), which all in conjunction are referred to as "thymic stroma". Mesenchymal cells are important supporters of thymus development and T cell differentiation in the fetal thymus (Itoi et al., 2007b). Myeloid cells, mainly macrophages and DCs, are necessary for apoptotic thymocyte clearance and central tolerance establishment, respectively (Surh and Sprent, 1994) (Oh and Shin, 2015). But the epithelial component of the thymic stroma, composed by TECs, is the essential element for T cell development (Anderson and Jenkinson, 2001) (Gray et al., 2005). TECs are responsible of progenitor homing to the fetal thymic anlage and of T cell specification via Notch signaling (Harman et al., 2003) (Koch et al., 2008); as direct contact of Notch ligand-expressing TECs with thymocytes is necessary to activate and sustain Notch signaling in developing thymocytes (Anderson et al., 2001) (Schmitt et al., 2004). Importantly, Notch signaling delivery by TECs is only sustainable when they are organized three dimensionally (3D) (van Ewijk et al., 1999), and disruption of the 3D architecture of TECs immediately results in downregulation of Notch ligand expression (Mohtashami and Zuniga-Pflucker, 2006). TECs comprise an heterogeneous population of cells (de Maagd et al., 1985) and 3D TEC organization allows their compartmentalization in specific niches, each responsible for specific stages of T cell development (Ladi et al., 2006). In general terms, TECs comprise two major populations, cortical (cTECs) and medullary (mTECs), both

derived from a common thymic epithelial progenitor cell (TEPC) (Bennett et al., 2002) (Rossi et al., 2006) (Bleul et al., 2006) (Baik et al., 2013) (Alves et al., 2014) of endodermal origin (Le Douarin and Jotereau, 1975). cTECs drive the early stages of T cell development, while mTECs are required for central tolerance establishment (Mizuochi et al., 1992) (Farr and Rudensky, 1998).

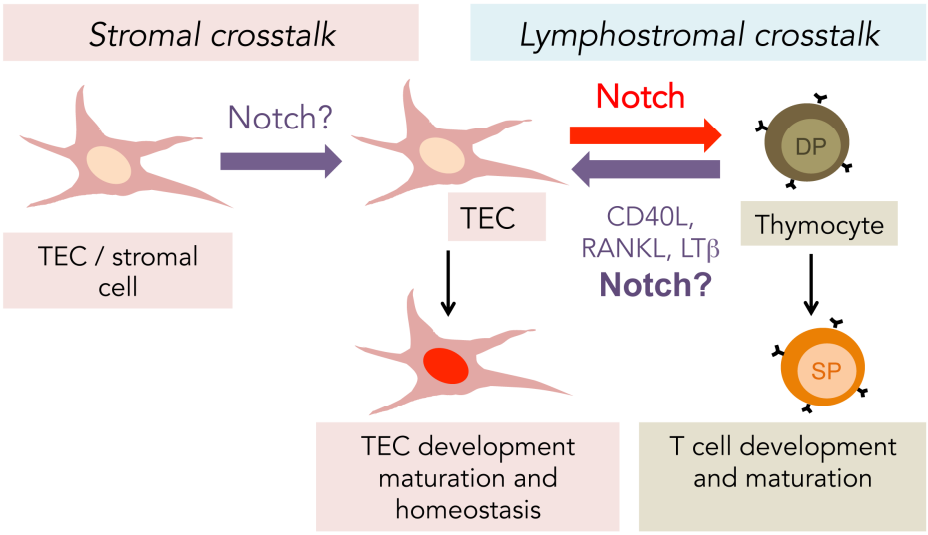


Figure 5. Thymic crosstalk. Thymocytes and stromal cells (mainly TECs) signal to each other in other to promote their mutual development, maturation or homeostasis via Notch signalling or the TNF receptor family.

The development and maturation of TECs and thus their hematopoietic capacity depends on the activity of the epithelial-specific transcription factor FoxN1 (Corbeaux et al., 2010; Nehls et al., 1994) (Gordon et al., 2001) (Itoi et al., 2007a). FoxN1 is required for differentiation of TEPCs into cortical and medullary TECs (Blackburn et al., 1996) (Bleul et al., 2006), although it is not completely known if it is required for the generation of the bipotent TEPC itself, as the FoxN1 *null* thymic primordium is able to separate from the parathyroid primordium and form and epithelial alymphoid thymic anlage (Nowell et al., 2011). But FoxN1 is not the only molecular cue required for TEC development. TECs support T cell development, but they are supported in turn by developing thymocytes. Indeed, although cTEC versus mTEC cell fate decision occurs in the absence of lymphocytes (Jenkinson et al., 2005), CD4+CD8+ double positive (DP) immature thymocytes are needed for successive cTEC differentiation. Likewise, mTECs require interaction with mature single positive (SP) cells for functional maturation (Philpott et al., 1992) (van Ewijk et al., 1994) (Shores et al., 1994) (Hollander et al., 1995) (van Ewijk et al., 2000). Such reciprocal interdependency between thymocytes and TECs is referred to as “thymic crosstalk” (Fig. 5). Unfortunately, the identity of the molecular signals derived from thymocytes to TECs remains

elusive. Recent studies showed that TNF receptor family signaling play a pivotal role in mTEC development (Rossi et al., 2007) (Akiyama et al., 2008) (Hikosaka et al., 2008), but little is known about the involvement of Notch signaling in this thymic crosstalk (see section 3.3).

2. T lymphocytes.

Among the lymphoid cell populations of the hematopoietic system, T cells are the cells responsible for scanning the intracellular environment for foreign invaders, killing infected cells, eradicating cancer cells, activating other immune cells and remembering antigens challenged in the past. Their immunological features are also responsible for rejection of transplanted organs, tissues and cells, and for the generation of nearly all autoimmune diseases and allergies, so their essential role in human immunity is irrefutable.

T cells can be divided into two major subtypes according to the polypeptide chains that form their T-cell antigen receptors: αβ T cells and γδ T cells (Fig. 6). Of them, γδ T cells recognize non-peptide antigens on intact proteins, while cells bearing the αβ TCR only recognize MHC-bound antigens presented by an antigen-presenting cell (APC). Both cell types are generated and mature within the thymus during a complex journey throughout specific intrathymic niches, which involves binary fate decisions orchestrated mainly by Notch signaling. However, little is known about the identity of *in vivo* niches that host TCRαβ and TCRγδ cells and their progenitors, the availability of Notch ligands and receptors in those niches, and the specific molecular requirements for their preferential generation and expansion.

2.1. The intrathymic development of αβ T cells is niche- and Notch- dependent.

T cells are generated in the thymus by a microenvironment-guided process in which thymocytes dynamically interacts with different thymic stromal cells that deliver Notch and other molecular cues at discrete steps of development (Ciofani and Zuniga-Pflucker, 2007) (Petrie and Zuniga-Pflucker, 2007) (Ladi et al., 2006). T cell development can be divided into two broad phases: an early phase, that takes place at the cortex, characterized by the expansion and differentiation of T cell precursors towards CD4+CD8+ double positive (DP) thymocytes, and a second late phase, that takes place at the medulla, in which immature DP thymocytes bearing the αβ TCR mature to CD4 or CD8 single positive (SP) T cells. During this process, each precursor that enters the thymus gives rise to approximately one million

progeny (Shortman et al., 1990). Five of the six major stages of T cell development occur at the cortex; and thus the cortex may contain several microenvironments different enough to promote sequential developmental changes. However, scarce reports have accounted for such cortical differences (Griffith et al., 2009).

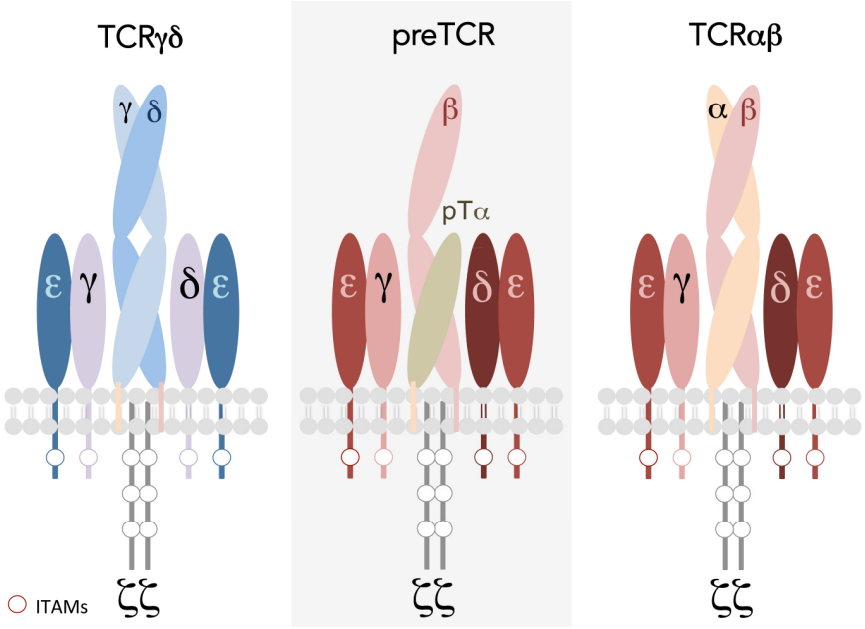


Figure 6. Molecular structure of the human T cell receptors. The signalling strength model claim that αβ/γδ T cell fate is determined by the strength of the signal delivered by the preTCR or the γδTCR. There are differences between both TCR complexes that might confer them differential signalling capacities at early stages of T cell development. The preTCR possess the three CD3 polypeptide chains (γ, δ, ε and ζ) and a pTα, while TCRγδ lacks pTα (Carrasco et al., 2001). In mice, TCRγδ also lacks CD3δ (Hayes and Love, 2002). Additionally, their expression levels at the plasma membrane differs notably, with the γδTCR being expressed in far greater numbers than the preTCR.

T cell development at the cortex:

The first steps of the early phase of T cell development involve the commitment of HPCs to the T cell fate in the thymus. Data from different groups, including ours, define the earliest human T cell progenitors or ETPs as c-kit+ CD34hi CD38+ CD1a- IL7Rlo (LSK-CD44+CD25- in mice) multipotent cells (Galy et al., 1993) (Andrews et al., 1989) (Marquez et al., 1998) that comprise about 0.03% of neonatal thymocytes, which are included within the most immature DN intrathymic population (DN1). ETPs lack self-renewal capacity and thus differ from the HSC compartment present in the BM (Galy et al., 1993) (Galy et al., 1995) (Allman et al., 2003), but they are similar to BM lymphomyeloid multipotent progenitors (LMPPs), as they retain T, NK, and myeloid lineage potential, and even display some B cell lineage potential (Porritt et al., 2004) (Garcia-Peydro et al., 2006) (Weerkamp et al., 2006)

(Bell and Bhandoola, 2008) (Luc et al., 2012) (Adolfsson et al., 2005). Therefore, ETPs may derive from BM LMPPs that enter the thymus through the CMJ, where they briefly stay as DN1 cells (Lind et al., 2001) and rapidly migrate upwards to the subcapsular cortex while interacting with cortical thymic stroma (Fig. 7) (Witt and Robbins, 2005). During this process, ETPs further differentiate along two alternative pathways through separate intermediate lymphoid or myeloid progenitors, characterized by differential expression of CD44 and IL7R. The CD34+ CD44lo IL7Rhi lymphoid progenitor (DN2 stage) is devoid of myeloid potential and comprises a bipotent population able to generate NK and T cells (Marquez et al., 1995) (de Yebenes et al., 2002). Cortical transmigration coincides with loss of non-T lineage potential, accessibility of TCR-γ, -δ , and -β loci (Petrie and Zuniga-Pflucker, 2007), a proliferation boost (Lind et al., 2001), and a developmental progress along successive DN2 and DN3 stages, the latter including CD4+ immature single positive thymocytes (CD4ISP) in humans (Kraft et al., 1993) (Hori et al., 1991). The important proliferation burst at the DN1 and DN2 stages is the consequence of several molecular cues provided by the TME, including c-kit, Wnt, Hedgehog (Griffith et al., 2009) and, most importantly, Notch1 and IL-7 (Peschon et al., 1994) (Balciunaite et al., 2005). Notch1 induces cell proliferation at early stages of T cell differentiation by upregulating IL7R in developing thymocytes (Gonzalez-Garcia et al., 2009). But the most important role of Notch1 in early intrathymic development is the induction of T cell lineage specification and the blockade of non-T cell fates (Schmitt et al., 2004) (Petrie and Zuniga-Pflucker, 2007) (Sultana et al., 2010). This occurs at the CMJ and is phenotypically characterized in humans by the downregulation of CD34 and CD44 (Marquez et al., 1995), the acquisition of the CD1a marker (Galy et al., 1993) and the transition to the DN3 stage (CD4ISP in humans; CD44-CD25+ in mice) (Fig. 7). In the absence of ligand-induced Notch signaling, T cell generation is severely impaired, as either Notch1 or Dll4 knock-out (KO) leads to complete absence of T cells (Radtke et al., 1999) (Koch et al., 2008) (Hozumi et al., 2008). Conversely, overexpression of Notch1 constitutively active intracellular form (ICN1), allow the ectopic generation of T cells at the BM (Pui et al., 1999). Other Notch receptors such as Notch2 or Notch3 are also able to induce T cell development but they are not essential *in vivo* (Suliman et al., 2011) (Waegemans et al., 2014).

Activation of Notch1 in human ETPs triggers the generation of CD34+ CD44lo IL7R+ lymphoid intermediates (DN2), while suppressing the generation of CD34+ CD44hi IL7R- CD123+ myeloid intermediate progenitors (Garcia-Peydro et al., 2006) (Martin-Gayo et al. *submitted*). According to current models, early Notch1 engagement by intrathymic Notch ligands blocks B cell potential in the earliest incoming LMPP progenitors (Harman et al., 2003), while other studies suggest that the T/B lineage choice occurs prior to

intrathymic Notch signaling, at least during fetal life (Harman et al., 2005) (Masuda et al., 2005). In any situation, continuous Notch signaling is required for intrathymic progenitors to progress along the T cell lineage (Schmitt et al., 2004) (Billiard et al., 2011).

Once thymocytes are committed to the T cell lineage, a first vawe of RAG gene upregulation (Wilson et al., 1994) initiates the rearrangements at the TCRγ, δ and β loci that finally result in the expression of either a mature γδ TCR or a pre-TCR (Groettrup et al., 1993). Pre-TCR expression coincides with the arrival of developing thymocytes to the SCC (Fig. 7), the simultaneous acquisition of CD4 and CD8 (DP stage) (Lind et al., 2001) (Kreslavsky et al., 2012) and the accessibility of the TCRα locus to the recombinase (Petrie et al., 1995). Productive rearrangement and expression of a TCRβ chain represents a survival signal for developing thymocytes, which are rescued from cell death by means of expression of a surface preTCR complex composed of TCRβ and the invariant pTα chain associated with CD3 components (Fehling et al., 1995) (von Boehmer and Fehling, 1997) (Ciofani and Zuniga-Pflucker, 2005). Pre-TCR signaling also induces a second intrathymic proliferation burst (Penit, 1988). These processes, known as β-selection also involve a directional change in the intrathymic migratory pathway of developing thymocytes, as β-selected thymocytes located at the SCC rapidly turn back inwards the cortex (Lind et al., 2001) (Fig. 7). Notch signaling also participates at this critical checkpoint, as pre-TCR-mediated β-selection critically requires Notch activation (Wolfer et al., 2002) (Balciunaite et al., 2005) (Maillard et al., 2006). Only after β-selection, thymocytes become independent of Notch signaling (Taghon et al., 2009), and immature DP thymocytes start to rearrange the TCRα locus (Carrasco et al., 1999) (Starr et al., 2003), which move randomly in the cortex as shown by *in vitro* real-time imaging of intact murine thymic lobes (Bousso et al., 2002) (Witt and Robbins, 2005). This random motility represents the dynamic consequence of TCR engagement of MHC-II-peptide complexes displayed by TECs at the cortical microenvironment. TCR engagement results in rescue from programmed cell death of DP thymocytes and acquisition of their definitve CD4+ or CD8+ single positive (SP) phenotype, a process known as positive selection; while DP thymocytes with no affinity for the MHC-II-peptide complexes expressed by cTECs die by neglect.

T cell development at the medulla:

Once DP thymocytes have been positively selected, CCR7 is upregulated on the cell membrane. CCR7 expression induces a rapid and directed migration from the cortex to the thymic medulla (Fig. 7), where mTECs express the CCL19 and CCL21 ligands (Ueno et al., 2004) (Witt and Robbins, 2005). At the medulla, positively selected TCRαβ+ thymocytes

undergo the process of negative selection by engaging self-antigens presented by mTECs and medullary DCs; therefore, the migration of positively selected thymocytes to the medulla is essential for the establishment of central tolerance. Finally, mature SP TCRαβ+ thymocytes egress the thymus from medullary and corticomedulary veins to the periphery.

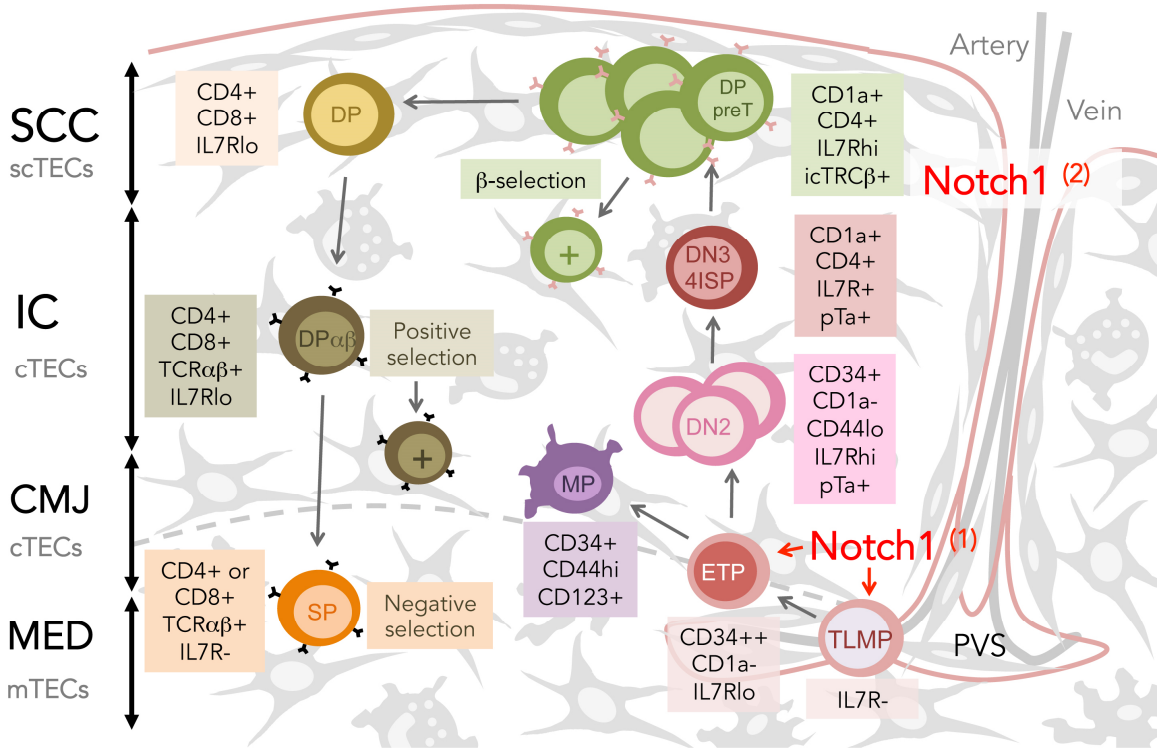


Figure 7. Intrathymic dynamics of T cell development. The image represents the intrathymic journey of developing thymocytes and how Notch signalling regulates T cell development in a microenviroment-dependent manner: (1) Notch-mediated inhibition of non-T cell lineages and proliferation; (2) Notch cooperates with the preTCR during β-selection. Double-pointed arrows at the left delimitate histologic thymic regions. Coloured boxes indicate the phenotype of each corresponding population. *TLMP:* Thymic lymphomyeloid progenitor; *ETP:* Early thymic progenitor; *MP:* myeloid progenitor; *icTCRβ:* intracellular TCRβ chain; *SCC:* subcapsular cortex; *IC:* inner cortex; *CMJ:* corticomedullary jucntion; *MED:* Medulla; *PVS:* perivascular space. Adapted from (Lind et al., 2001).

2.2. Models of *in vitro* T cell development.

An efficient immune reconstitution of the T-lymphoid compartment in immunocompromised patients represents a major clinical challenge. *De novo* generation of T cells from donor BM stem cells may take up to 12 months in transplanted patients, period in which opportunistic infections, complications as GvHD and the need of continuous supply of cytokines, compromise the survival and/or expansion of the cellular allograft (Andre-Schmutz et al., 2009). Due to those limitations, *in vitro* development and large-scale expansion of TCRαβ and TCRγδ cells suitable for human immune reconstitution is one of the main goals of modern Immunology.

With the exception of T cells, any hematopoietic lineage can be generated *in vitro* by cytokine stimulation or co-culture on stromal feeding layers (Rolink et al., 1995) (Vuckovic et al., 2002) (Luevano et al., 2012). But the complexity of T cell development established the idea of a compulsory requirement for an intact 3D thymic structure, as 2D TEC monolayers were incapable of mimicking the TME (Montecino-Rodriguez et al., 1996). Latter on, Notch signaling activation was identified as sufficient to induce T cell fate on HPCs. Nevertheless, additional stromal-derived signals were required to support full differentiation of T cells, as ETPs overexpressing ICN1 are unable to undergo β-selection unless co-cultured with stromal cells (Garcia-Peydro et al., 2006). Due to these limitations, fetal thymic organ culture (FTOC) was initially envisioned as the only culture system matching both requirements. But in 2001, elegant experiments initiated by Parreira and coworkers using S17 murine stromal cells (Jaleco et al., 2001) and followed by Zuñiga-Pfucker (Schmitt and Zuniga-Pflucker, 2002) using the murine bone marrow OP9 stromal cell line (Kodama et al., 1994), showed that those BM stromal cells, which efficiently support B (Vieira and Cumano, 2004) and NK cell generation (Williams et al., 1999) from murine HPCs *in vitro*, acquire the ability of generating T cells when they ectopically express the Notch ligand Dll1 (Fig. 9) (Schmitt and Zuniga-Pflucker, 2002) (De Smedt et al., 2004) (La Motte-Mohs et al., 2005) (Zuniga-Pflucker, 2004) (de Pooter and Zuniga-Pflucker, 2007). While this phD thesis was in progress other Notch ligands including Dll4, Jag1 and Jag2, were shown to display the ability to support *in vitro* T cell development, although with different efficiencies (see section 3.2 and Results II).

While in general terms, the Notch ligand expressing-OP9 *in vitro* co-culture assay is currently envisaged as an efficient system for generating αβ T lineage cells, there are still some important limitations regarding MHC restriction and thus generation of mature T cell populations. Specifically, OP9 cells do not express MHC molecules (Gao et al., 2010), only

IFNγ stimulation induces endogenous MHC-I expression (Van Coppernolle et al., 2009), thus MHC-restricted human CD8+ T cells suitable for clinical purposes might not be generated in any case. Nevertheless, generation of innate-like T cells and minute amounts of functionally mature CD4+ and CD8+ TCRαβ cells has been reported to occur in OP9-Dll1 co-cultures, via MHC-I expressed by developing thymocytes (Van Coppernolle et al., 2009) (Awong et al., 2011). Besides, more physiological approached using human TEC cell lines have been also considered. But TEC cell lines were able to support T cell development only when retrovirally transduced with the Dll1 ligand (Beaudette-Zlatanova et al., 2011), experimental procedure that discards the suitability of the mature T cell generated for the clinic. New approaches devoid of feeder layers might be the promise of large-scale *in vitro* T cell generation. Besides, αβ T cells, γδ T lineage cells can also be observed in these co-culture assays, but Notch requirements for large-scale *in vitro* expansion of γδ T cells are not well understood yet, and contradictory results have been obtained in mice and humans (see section 2.4).

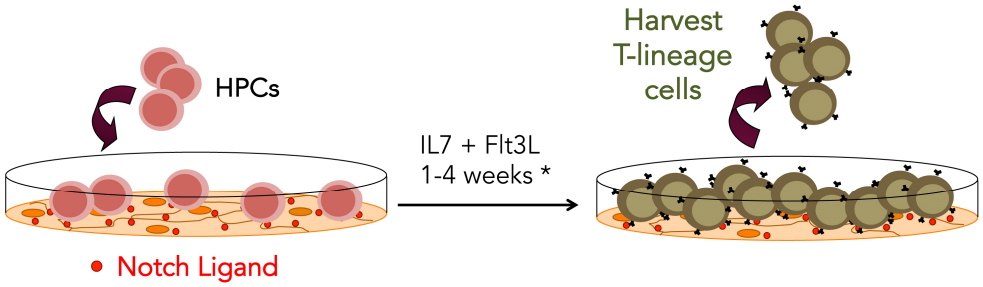


Figure 8. Co-culture system of HPCs with Notch ligand-expressing OP9 stromal cells for *in vitro* T cell development. HPCs are seeded onto monolayers of Notch ligand-expressing OP9 cells, and induced to differentiate in the presence of exogenous IL-7 and Fms-related tyrosine kinase 3 ligand (FLT3L). After 1 to 4 weeks of co-culture, resulting T-lineage cells are harvested for further applications of phenotypic analysis. (*) The length of the culture period depends on the desired stage of T-cell differentiation that is to be studied.

2.3. Gamma-delta T cells.

The relative "recent" discovery of T cells bearing a different antigen receptor than classical αβ T cells (Saito et al., 1984) (Pardoll et al., 1987), together with its rare occurrence in humans and mice have traditionally consigned γδ T cell research to a second place with respect to TCRαβ research. But in the last decade, immunologist have appreciated that γδ T cells represent a promising and intriguing new aspect of immunology by their unique antigen recognition patterns and special immunological properties. Notably, these special properties fit in none of the two classical branches of Immunology, the innate and the adaptive, being mostly a "bridge" between the two (Vantourout and Hayday, 2013). The γδ TCR directly recognizes antigens from intact proteins or non-peptide compounds, while αβ TCRs only

recognize MHC-bound antigens on an APC. Indeed, αβ T cell development and maturation is MHC-dependent while γδ T cell development seems to be independent and γδ thymocytes mature just after high affinity engagement of the γδ TCR with a self ligand present in the thymus (Xiong and Raulet, 2007).

Although both cell types emerge in peripheral blood (Campbell et al.) of most vertebrates, γδ T cells are rare in murine and primate PB, representing about 5% of mononuclear cells. Conversely, they constitute a major T-cell population at other anatomical locations such as the gut mucosa and other epithelial tissues (O'Brien et al., 2007) (Hua et al., 2013). As αβ T cells, human γδ T cells differentiate in the thymus from pluripotent CD34+ ETPs (Dudley et al., 1995) (Kang and Raulet, 1997) (Garcia-Peydro et al., 2003), where they comprise about 1% of total thymocytes (Offner et al., 1997). As in mice, the particular γδ TCRs expressed by human fetal γδ thymocytes are distinct from those expressed in postnatal life (Garman et al., 1986) (Chien et al., 1987) (Havran and Allison, 1988), a finding that raised the confirmed idea that γδ T cells are produced in ordered waves that populate different anatomical sites. In humans, early fetal rearrangements at the TCRδ locus initially involve Vδ2 segments. Later, there is a switch to Vδ1, implying that Vδ1+ and Vδ2+ subsets arise via rearrangements occurring at distinct phases of thymic ontogeny (Krangel et al., 1990). In contrast with αβ T cells, a β-selection-like process for γ or δ chains has not been found, and selective rearrangements at different moments during ontogeny may represent a mechanism for γδ T cell diversity in specific tissues and PB (Lauritsen et al., 2006).

The anatomical location of γδ lymphocytes has profound implications for their antigen specificity. For example, 50% to 90% of human γδ T cells in PB use a combination of Vγ9 and Vδ2 gene segments (Allison et al., 2001). These human Vγ9/Vδ2 T cells, are unique in that they specifically respond to a set of non-peptidic phosphoantigens, highly expressed by bacteria and cancer cells (Riganti et al., 2012), and by the secretion of IFN-γ and TNF-α. Their potent anti-tumor activities have prompted the development of protocols of adoptive immunotherapy of autologous *in vitro*-activated TCRγδ cells and new treatments in which TCRγδ-agonists are administered to cancer patients (Meraviglia et al., 2010) (Castella et al., 2011) (Deniger et al., 2014). Similarly, TCRγδ Vδ1 cells are the object of recent interest (Bruno Silva-Santos, 4th European Congress of Immunology. September, 2015). They are more prevalent in tissues than in PB and interact mainly with MHC-related antigens, as CD1c, CD1d MIC-A, MIC-B and ULPBs (Spada et al., 2000) (Groh et al., 1998) (Cosman et al., 2001). Thus, it is well established that γδ T cells comprise heterogeneous subsets with different tissue-specific functions that increase their antigen recognition range.

Despite their functional importance, the intrathymic dynamics of γδ T cells and the several differentiation stages through which they develop remain unclear, especially in humans. In mice, several studies have demonstrated that γδ T cells differentiate in the thymus along a DN pathway (Fisher and Ceredig, 1991) (Pennington et al., 2005), in contrast with TCRab cells that differentiate along a DP pathway. Besides, the expression of other cell-surface markers including CD24 (or heat-stable Ag; HSA), distinguishes immature from mature TCRγδ thymocytes, as CD24 becomes downregulated during γδ lineage maturation (Tatsumi et al., 1993). Similarly, the immature αβ lineage marker CD1a (Galy et al., 1993) (Res et al., 1997) that is expressed in 50 to 90% of human postnatal γδ thymocytes can be used to distinguish immature human γδ thymocytes from functionally mature γδ T cells (Offner et al., 1997) (Van Coppernolle et al., 2009) (Van Coppernolle et al., 2012). However, in contrast to mice, γδ T cells differentiate along a DP pathway in the human postnatal thymus. Accordingly, the human postnatal thymus contains DN, DP, CD4+ and CD8+ SP γδ T cell populations (Strominger et al., 1989) (Van Coppernolle et al., 2012), thus traditional characterization of human γδ T cells based on lack of CD4 and CD8 expression is not accurate. Indeed, DP and CD4+SP γδ populations represent the majority of human intrathymic γδ T cells. These subsets are only found in the thymus, as peripheral γδ T cells are either DN or CD8+SP (Groh et al., 1989). Nevertheless, γδ T cells expressing CD4 and/or CD8 can be seen in the periphery in pathological conditions, such as in patients with an atypical CD3δ immunodeficiency, in which CD4+ γδ T cells are 10-fold enriched in comparison with normal individuals (Garcillan et al., 2014). Also, the majority of γδ T cells found in the PB or BM of γδ T-ALL patients are heterogeneous in their CD1/CD4/CD8 phenotype, and display either a CD4+SP or a DP phenotype (Falini et al., 1989) (Langerak et al., 1999).

The progenitor-progeny relationships between all those four human intrathymic γδ developmental stages is still a matter of debate, although recently, Van Coppernolle et al. have demonstrated that immature CD1a+DP γδ thymocytes can give rise to mature CD1a-SP γδ T cells *in vitro*. However, the DP stage is not a compulsory developmental stage in the absence of Notch signaling, this implying that both populations may arise independently from a common CD1a+ DN TCRγδ+ precursor (Van Coppernolle et al., 2012). The observation that Notch signaling supports the differentiation of human γδ progenitors along a DP pathway *in vitro*, point to the importance that microenvironmental signals provided by intrathymic niches to γδ T cell progenitors may have during their development in the thymus. However, unlike αβ T cell development, the characterization of the intrathymic niches that host the

development of γδ thymocytes as well as their intrathymic dynamics are poorly understood. It is known that human γδ T cells are mainly located at the thymic medulla, although some are also found in the cortex (Falini et al., 1989), and that CCR7-directed migration through different intrathymic niches controls their development in mice (Reinhardt et al., 2014), so step-wise delivered signals from particular thymic miroenvironments may be important for human γδ T cell development, as they are for development of αβ T cells.

2.4. αβ versus γδ lineage specification.

Soon after T lineage commitment, developing thymocytes split from a DN bipotent precursor into αβ- or γδ-lineage T cells depending on productive rearrangements and expression of *TCRG*, *TCRD* and *TCRB* genes (Petrie et al., 1992). This choice is controlled at the transition between late DN2 and DN3 stage (Rothenberg, 2014) but the molecular mechanisms involved are still controversial. Two classical models of T cell fate determination were early proposed, the instructive and the stochastic model (Kang and Raulet, 1997) (MacDonald and Wilson, 1998). The instructive model gives a primary role to the TCR and proposes that successful expression of either TCRγ and TCRδ or TCRβ polypeptide chains determines specific lineage commitment (Alison and Lanier, 1987; Pardoll, 1987) (Livak et al., 1995). Alternatively, the stochastic (or selective) model proposes that cell fate is determined independently and prior to TCR rearrangements, giving a secondary role to the TCR in cell fate determination just as a driver of survival and proliferation of already pre-commited thymocytes (Terrence et al., 2000). Although evidence exists to support either model (Kang et al., 1998) (Sherwood et al., 2011) (Ishida et al., 1990) (Schweighoffer and Fowlkes, 1996), a reviewed version of the instructive model, the "signaling potential" model (Fig. 9), posits that the strength of signal transduced by the TCR expressed by late DN2-DN3 thymocyte dictates its lineage specification (Robey, 2005) (Pennington et al., 2005) (Kreslavsky et al., 2008). Consequently, strong signals, usually delivered by the gd TCR, drive commitment towards the gd lineage, while weak signals, usually delivered by the preTCR, promote the ab T cell fate (Hayes and Love, 2002) (Hayes et al., 2005) (Haks et al., 2005) (Li et al., 2010) (Fig. 9). However, the identity of the expressed TCR in uncommitted progenitors is not important for fate outcome, as *in vitro* manipulation leading to a γδ TCR weakened signal may instruct the αβ T cell fate (Zarin et al., 2014), while an early expression of the αβTCR or a strong signal delivered by the preTCR can replace the γδ TCR in the development of γδ lineage cells in an Id3-dependent manner (Bruno et al., 1996) (Terrence et al., 2000) (Haks et al., 2005) (Lauritsen et al., 2006) (Kreslavsky et al., 2008).

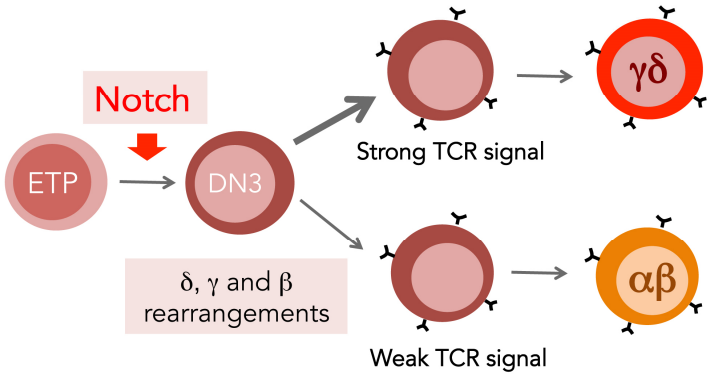


Figure 9. Signalling potential model of αβ/γδ T cell lineage specification. Reviewed instructive model of αβ/γδ T cell fate specification considering the strength of the signal delivered by the TCR and the role of other instructive signalling pathways as Notch signalling. Lineage decision is made depending on TCR signal strength, no matter what kind of TCR is expressed by the T cell precursor. A strong TCR signal will drive γδ T cell development, while weak TCR signal will lead progenitors towards the αβ-lineage. Notch signalling pathway also instruct αβ/γδ T cell outcome at early time points when the TCR is not yet expressed. Modified from (Garbe and von Boehmer, 2007).

Besides the apparently well-established dependency of the αβ/γδ lineage determination from the TCR, there are indications that the TCR is not the only player. Several evidences point to Notch and its IL7R target as important players in the process (Washburn et al., 1997). IL7R signaling regulates the accessibility to the TCRγ locus (Huang et al., 2001) (Ye et al., 2001), and T cell progenitors with high IL-7R expression levels seem to be biased towards the γδ lineage in mice (Kang et al., 2001). Conversely, absence of ligand engagement by Notch receptors favors γδ over αβ T cell generation, as the latter compulsory require Notch signaling (Ciofani et al., 2006) (Tanigaki et al., 2004) (Washburn et al., 1997) (Garbe et al., 2006). Indeed, BM progenitors heterozygous for a Notch1 inactivating mutation, or deficient in RBPjκ, develop almost exclusively to γδ T cells (Tanigaki et al., 2004; Wolfer et al., 2001); while constitutively active Notch1 induces murine γδ thymocytes to adopt the αβ T cell fate (Washburn et al., 1997). Intriguingly, γδ T cell numbers are 2-fold reduced in the fetal thymus of Jag2-deficient mice (Jiang et al., 1998), a finding that concurs with results in humans showing that Notch signaling favors γδ T cell generation at the expense of αβ T cells in fetal thymic organ culture (FTOC) (Garcia-Peydro et al., 2003) (De Smedt et al., 2002). Indeed, high Notch activity in human intrathymic progenitors increases *DTX1*, *NRARP* and *RUNX3* gene expression, genes that are downregulated during αβ-lineate differentiation (Van de Walle et al., 2009). As a whole, these controversial results suggest that small differences in Notch signaling strength in particular contexts or developmental stages (Wolfer et al., 2001)

are translated into different cell fate outcomes as confirmed by modulation of Notch activation with a γ-secretase inhibitor (GSI) (Doerfler et al., 2001) (Van de Walle et al., 2009), or as a result of different Notch receptor-ligand interactions (Van de Walle et al., 2013). Importantly, these results may also reflect the non-redundant roles of individual Notch receptors and ligands, whose expression in particular thymic niches may differ from human to mice (Van de Walle et al., 2013) (Jaleco et al., 2001). Therefore, one of the aims of this study has been to assess whether context-specific changes in Notch-signaling activity may coordinate the αβ/γδ lineage decision during intrathymic development (Guidos, 2006) (Ciofani et al., 2006) (Garbe and von Boehmer, 2007).

2.5. Molecular signatures of T cell specification and αβ versus γδ T cell lineages.

Hematopoiesis is characterized by a progressive loss of developmental potentials that correlates with the activation of lineage-specific transcriptional programs. T cell development involves the Notch1-mediated inhibition of non-T cell lineages, which is the result of transcriptional repression of B and myeloid specific genes as C/EBPα, EBF1, PAX5 and PU.1. Conversely, Notch1 activation induces the expression of T cell lineage specific transcriptional program, including expression of IL7R, GATA-3, TCF-1, LEF-1, E2A or BCL11B, which in turn will later activate expression of genes involved in TCR assembly and signaling, as RAG, pTα or CD25 (Rothenberg, 2014) (Yui and Rothenberg, 2014). Once the T cell lineage is specified, progenitors must choose between two alternative fates: αβ or γδ, but a consensus about αβ/γδ lineage-specific transcriptional programs has not been reached as yet. It is known, though, that high Notch activity increases the expression of DTX1, NRARP and RUNX3 genes, which are down-regulated during αβ-lineage differentiation (Taghon and Rothenberg, 2008). Additionally, strong signaling transduced by the γδTCR leads to greater activation of the MAPK pathway, upregulation of Id3 and Egr family transcription factors (Robey, 2005) (Taghon et al., 2006), and thus the initiation of the γδ rather than the αβ T cell program; a finding that concurs with the fact that Id3 plays a prominent role in γδ fate determination but is dispensable for adoption of the αβ fate (Lauritsen et al., 2006).

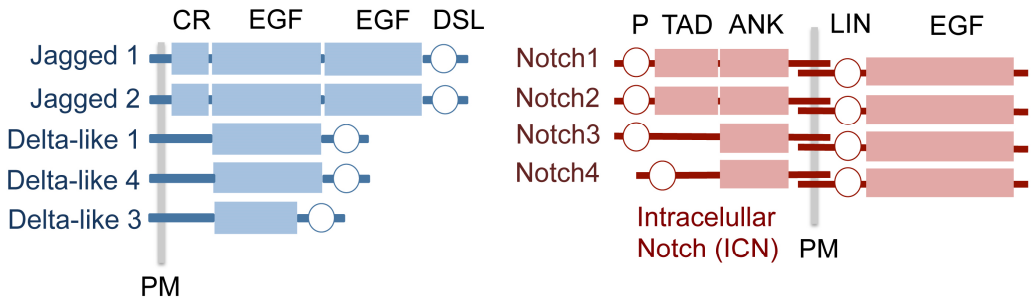
Recently, new gene signatures of the γδ T cell lineage have been identified in mice, including Etv5 and the TCF-1 related transcription factor Sry-like HMG-Box 13 (Sox13) (Melichar et al., 2007) (Jojic et al., 2013), which are both expressed in thymic precursors, and may thus be among the earliest determinants of the γδ lineage. But whether these factors are activated downstream of the γδTCR or by an inductive signal prior to TCR expression remains unclear.

2. The Notch signaling pathway.

We are about to celebrate the centenary of the discovery of Notch, an evolutionary conserved gene identified in 1917 by a mutant fly with "notches" in its wings (Morgan, 1917). Since then, Notch has been characterized as one of the key players during embryonic and adult development. Notch regulates cell fate and tissue patterning in many organisms, by controlling a huge diversity of key cellular processes as cell fate determination, proliferation, differentiation or cell death in many different tissues, including the hematopoietic system (Tanigaki and Honjo, 2007). Additionally, Notch activation plays a role in disease, as several cancer-related genes, including *MYC*, *CCND1*, *BCL2* or *CDKN1A* (Weng et al., 2006) are Notch direct targets; and Notch is itself mutated in different cancers, including more than 50% of T-cell acute lymphoblastic leukemias (T-ALL) (Weng et al., 2004).

3.1. Notch receptors, ligands and intracellular signaling.

Notch receptors comprise a highly conserved family of transmembrane heterodimeric receptors. In mammals, Notch family members include four receptors (Notch1, Notch2, Notch3 and Notch4) that may interact with up to five ligands, two from the Jagged family (Jag1 and Jag2) and three of the Delta family (Dll1, Dll3 and Dll4) (Fig. 11). Ligand binding induces the exposure of an extracellular site at the juxtamembrane heterodimerization domain (HD) susceptible of proteolitic cleavage by the ADAM-family metalloproteases, which promotes in turn, a second cleavage at the intracellular region by the γ-secretase complex, and releases the intracellular active form of the Notch receptor (ICN) required for T cell development (Doerfler et al., 2001). Next, ICN rapidly translocates into the nucleus were it interacts with the DNA-binding protein CSL (RBPjκ in mice) and its co-activator Mastermind (Mam) to activate the transcription of Notch target genes, including the conserved hairy/enhancer of split (*HES1*, *HES5* and *HES7*) family genes, the Hey subfamily (*HEY1*, *HEY2* and *HEYL*), *NRARP* or *DTX1* (Iso et al., 2003) (Fig. 11). In T cells, *CD25*, the *IL7RA, PTCRA* and the *GATA3* transcription factor gene are direct targets of Notch1 (Gonzalez-Garcia et al., 2009) (Reizis and Leder, 2002) (Deftos et al., 2000) (Fang et al., 2007). Interestingly, Notch family genes as *NOTCH1* and *NOTCH3* are themselves Notch targets, thus establishing a positive feedback activation loop (Ikawa et al., 2006) (Del Monte et al., 2007) (Van de Walle et al., 2009), which is a key feature of the pathway, as it amplifies small differences in Notch ligand and Notch receptors expression within equivalent cells. In the thymus, for example, transgenic mice expressing a constitutively active form of Notch1 upregulate the endogenous *NOTCH1* gene (Robey et al., 1996).



	Notch1	Notch2	Notch3	Notch4
Dll1	++ (3)	+ (2)	++ (3)	nd
Dll4	+++ (5, 6, 7)	nd	+ (6,9)	+ (1, 8)
Jag1	+ (3, 4, 6)	+ (2, 4)	+ (3, 4, 6)	+ (10)
Jag2	++ (3)	+ (2)	+++ (3, 6)	nd

Figure 10. Notch ligands and receptors: Molecular structure and Notch ligand – Notch receptor putative interactions. *A,* The structure of Notch receptors comprises an extracellular region with 29 to 36 EGF-like repeats (EGF) and three copies of a LIN12/Notch-motif (LIN), and an intracellular region containing Ankyrin repeats (ANK) and a PEST domain (P). The molecular architecture of Notch ligands includes an N-terminal MNL domain, a cysteine-rich DSL module and 6 to 16 EGF-like repeats. Jagged ligands also contains a cisteine-rich region (CR) preceding the transmembrane domain. *B,* Notch ligands and receptors expressed in the human thymus and their putative interactions. The solid-phase binding assays used to tests Notch ligands – Notch Receptor interactions may not indicate final affinity of each ligand to each receptor as binding affinities may be modified by Fringe proteins. (1) (Shawber et al., 2003); (2) (Shimizu et al., 2000a); (3) (Shimizu et al., 2000b); (4) (Shimizu et al., 1999); (5) (Andrawes et al., 2013); (6) (Van de Walle et al., 2013); (7) (Mohtashami et al., 2010); (8) (Yan et al., 2001); (9) (Indraccolo et al., 2009); (10) (Pedrosa et al., 2015) (11) (Shutter et al., 2000), (12) (Besseyrias et al., 2007).

Notch signaling is also involved in the maintenance of the stem cell pool in different adult tissues, as the hematopoietic system (see 3.2) and the gut (Pellegrinet et al., 2011), while in other contexts, such as in skin keratinocytes, Notch signals seem to promote differentiation (Lefort and Dotto, 2004). These differential Notch activation outcomes may depend on the spatial and temporal-restricted expression of: (1) the ligands and/or receptors, or (2) Notch receptor modulators (Bray, 2006). Indeed, many molecular mechanisms differentially regulate the pathway in a context-dependent manner, including post-translational modification of Notch receptors by Fringe glycosil transferases that modify their capability to respond to Delta ligands (Koch et al., 2003), and by Neuralized (Neur) and Mindbomb (Mib) ubiquitin ligases, that are required for ligand activation (Glittenberg et al., 2006) (Chitnis, 2006). Additionally, intracellular ligand trafficking that occurs upon endocytosis of ligand-engaged Notch receptors is an essential event for successful activation of Notch in the responding cell (Seugnet et al., 1997) (Nichols et al., 2007) (Kopan and Ilagan, 2009). More importantly, distinct Notch ligands and receptors have non-redundant functions and activate the Notch pathway differently (Shimizu et al., 2002) (de La Coste and Freitas, 2006). Several reports

show that Notch1 receptor interacts differently with Dll1, Dll4 and Jag1 ligands *in vitro*, and comparative binding studies show preferential interaction of Notch1 with Dll4, whereas binding of Notch1 to Dll1 (Besseyrias et al., 2007) (Andrawes et al., 2013) (Cordle et al., 2008b) or Jag1, is weaker (Cordle et al., 2008a). Notch ligands and receptors also show non-overlapping roles *in vivo*. For example, mature T cells developed in a Dll1- or Dll4-enriched environment show different profiles of cytokine production (de La Coste et al., 2005). Also, the Notch1-Dll4 interaction induces T cell lineage commitment in ETPs, while Notch2-Dll1 is capital for marginal zone B-cell development (Hozumi et al., 2004) (Saito et al., 2003). In the vascular system, the Notch1-Dll4 interaction induces angiogenesis (Hellstrom et al., 2007), while the Notch1-Jag1 axis counteracts this effect (Benedito et al., 2009). All these data demonstrate that besides Notch is a well-known signaling pathway, the outcome of Notch activation is very diverse and strictly dependent on multiple context-dependent factors (Pear and Radtke, 2003). Therefore, it is tempting to speculate that such context-dependent factors may well reflect the regulation of signals provided by distinct ligand-receptor complexes, due to their differential spatial and temporal regulation during a particular developmental process. Whether this regulatory mechanism actually controls the generation of T cells in the human thymus represents an important aim investigated in this study.

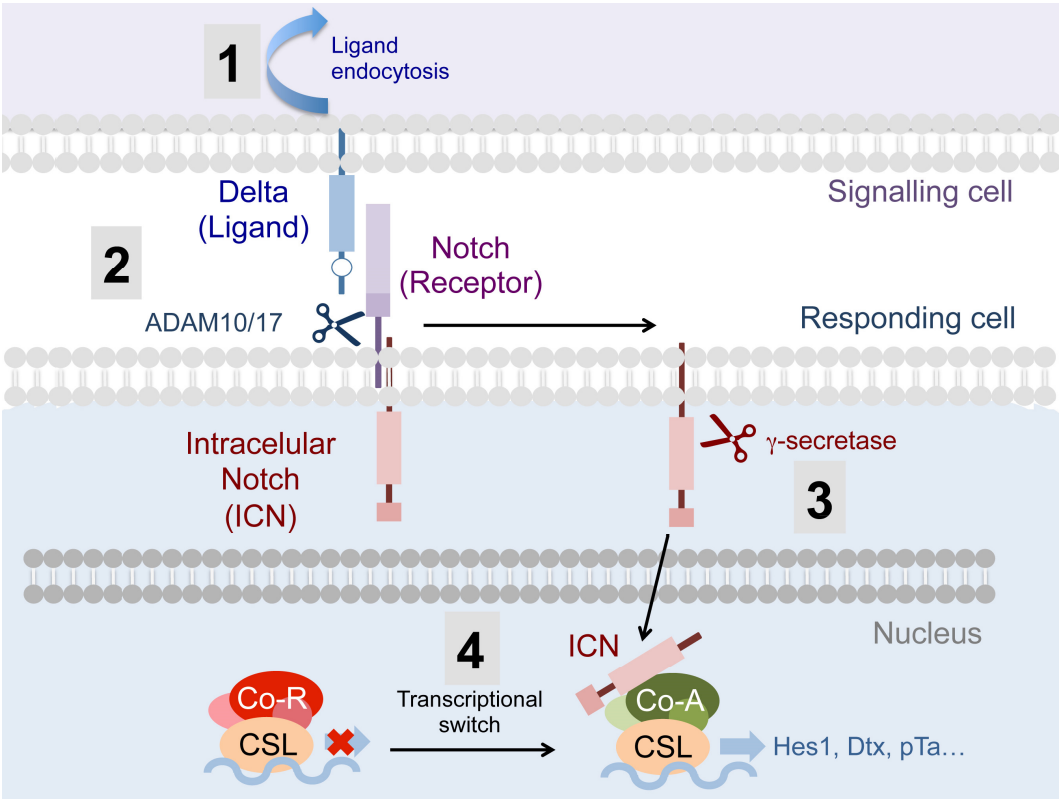


Figure 11. The Notch signalling pathway. (1) Notch ligand – Notch receptor engagement: The ligand is endocytosed by the signalling cell generating a pulling force that expose de ADAM10/17 cleavage site of the receptor (2). ADAM10/17 cleavage induces second cleavage by the γ-secretase complex (3) that releases the intracellular active portion of the Notch receptor (ICN), which translocate to de nucleus and activate transcription (4). Adapted from (Kopan and Ilagan, 2009).

3.2. Role of Notch signaling in hematopoiesis.

Notch1 activation is essential for HSC generation during embryonic development (Kumano et al., 2003) and seems to be involved in lineage specification at particular developmental branch points such as T versus B (Izon et al., 2002) (Koch et al., 2001) (Pui et al., 1999) (Radtke et al., 1999), αβ versus γδ (Washburn et al., 1997) (Van de Walle et al., 2013) and CD4 versus CD8 (Robey et al., 1996) cell fate decisions. But T cell fate specification is the most prominent function of Notch in hematopoiesis, as loss-of-function of Notch1 in mice results in a complete block in T cell development at the DN stage accompanied by an ectopic emergence of B cells in the thymus (Radtke et al., 1999). Interestingly, mice lacking RBPjκ (Han et al., 2002) or bearing a Dll4 conditional deletion in TECs (Koch et al., 2008) (Hozumi et al., 2008) showed an identical phenotype, a finding that places Dll4 as the critical Notch ligand for *in vivo* T cell development. Conversely, Notch1 gain-of-function induced by ICN1 overexpression in BM imposes ectopic T cell development at the expense of B-cell development (Pui et al., 1999), and the same effect was observed after Dll4 overexpression in the thymus (Dorsch et al., 2002) (Fig. 12). Additionally, numerous *in vitro* studies have demonstrated that ligand-dependent activation of Notch on HPCs induce T cell commitment (see 2.2.) The mechanism of action of Notch signaling underlying differential outcomes at distinct developmental branch points seems to be quantitative rather than qualitative, as discussed above for the αβ/γδ lineage decision (see section 2.4). In fact, increasing thresholds of Notch signaling in CB progenitors sequentially suppress B, myeloid/DC an NK lineage fates in human and mice (De Smedt et al., 2005) (Garcia-Peydro et al., 2006) (Pui et al., 1999). In the thymus, Notch1 is preferentially expressed *in vivo* at the thymic cortex and Notch1 downregulation is required for further maturation of cortical thymocytes, this indicating that Notch activation is tightly regulated during thymopoiesis (Hasserjian et al., 1996) (Huang et al., 2003) (Radtke et al., 1999). These differential Notch activation levels can be provided by different Notch ligands. For example, *in vitro* T cell specification of human CD34+ CB progenitors (Jaleco et al., 2001) (Hozumi et al., 2004) or murine BM-derived HPCs (Lehar et al., 2005) is achieved through Dll1 or Dll4, but not by Jag1. Conversely, all Notch ligands support T cell development from ETPs (Besseyrias et al., 2007), suggesting that not only differential Notch-ligand induced signaling, but the progenitor developmental stage results in different outcome. This is because Jagged ligands seem to induce lower Notch1 activation than Delta ligands (Van de Walle et al., 2011), being Dll4 a more effective inducer of T cell differentiation than Dll1 at low expression levels similar to those observed *in vivo* in cTECs (Lefort et al., 2006) (Mohtashami et al., 2010), as Dll1 at low densities enhance the generation of B-lymphoid precursors (Dallas et al., 2005). Moreover, binding studies show preferential interaction of

Notch1 with Dll4 rather than with Dll1, which binds Notch1 weakly (Besseyrias et al., 2007). Thus a quantitative regulation of Delta expression levels within the TME or a differential expression of distinct Notch ligands at particular intrathymic niches could be responsible for the induction of a different Notch activation strength during thymopoiesis (Fiorini et al., 2008) (Billiard et al., 2011).

Regarding the intrathymic distribution of Notch ligands, several reports in mice have shown that Dll1, Dll4, Jag1 and Jag2 are differently expressed at singular thymic regions. For example Dll1 is abundant in the vasculature and at the CMJ (Schmitt et al., 2004) (Griffith et al., 2009), while Dll4 is abundantly present in cTECs specifically at the SCC (Hozumi et al., 2008) (Koch et al., 2008) (Mohtashami et al., 2010). Conversely, Jag1 is exclusively expressed at the medulla, and Jag2 mRNA has been detected only at the inner thymic cortex (Lehar et al., 2005), (Heinzel et al., 2007) (Mohtashami et al., 2010). But nothing is known about Notch ligand distribution in the human thymus. Among all Notch ligands present in the thymus, Dll4 is the essential and non-redundant ligand for T cell commitment at least in mice (Koch et al., 2008) (Hozumi et al., 2008). Conditional deletion of Dll4 *in vivo* in the murine thymus results in a complete abrogation of T cell development and the ectopic appearance of B cells (Koch et al., 2008); a phenotype similar to that obtained upon Notch1 abrogation (Radtke et al., 1999). On the contrary, Dll1 abrogation in TECs does not impair T cell development (Hozumi et al., 2004). Interestingly, Dll4 supports T cell lineage development exclusively via the Notch1 member of Notch receptors. Although Notch2-Dll1 interactions can also induce T cell lineage commitment, they do not support T cell maturation beyond the DN3 stage (Besseyrias et al., 2007). Thus, specific Notch ligands – Notch receptors interactions are required for successful T cell commitment and development (Fig. 10).

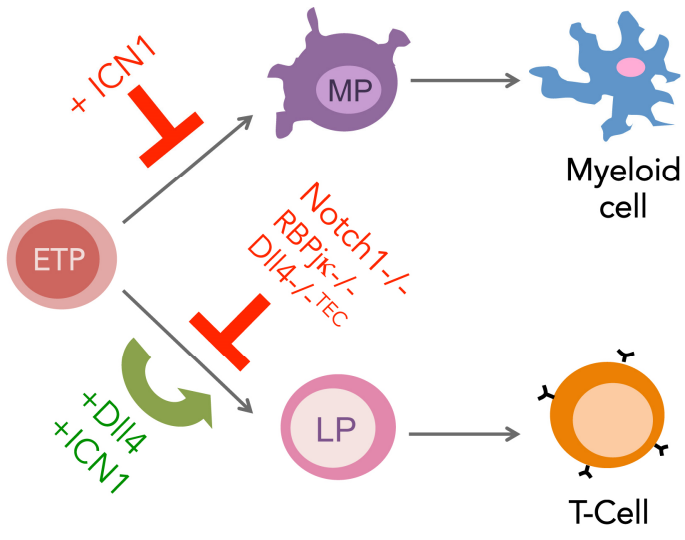


Figure 12. Notch1 and T cell fate specification. Loss of Notch function through Notch1 or RBPjκ abrogation in HPCs, or Dll4 in TECs, blocks T cell development. Conversely, gain of function of Notch signalling via over-expression of ICN1 or Dll4, favours the generation of the lymphoid progenitor, while blocks myeloid progenitor development. Notch mediated-inhibition of the myeloid potential is ligand specific, Dll4 is the essential Notch ligand for T cell specification, as Dll1 in low levels is not able to sustain T cell development *in vitro*.

3. 3. Role of Notch signaling in γδ T cell development and function.

As discussed above, Notch signaling may play a critical role in αβ versus γδ T cell fate determination once T cell fate has been specified, but whether Notch signaling also regulates further differentiation and function of γδ T cells after γδ T cell commitment has been induced is essentially unknown. As learned from αβ T cell development, Notch signaling must be delivered in a step-wise fashion during the intrathymic journey of γδ T cells, but little is known about the intrathymic niches that host γδ T cells *in vivo*. What is known from *in vitro* studies, is that the TCRγδ DP differentiation pathway is strongly dependent on Notch signaling (Van Coppernolle et al., 2012) and highly proliferative, while the TCRγδ DN pathway gives rise to functionally mature DN and CD8 SP γδ cells in the absence of Notch signaling. Thus, a capital role for Notch signaling in γδ T differentiation and functional maturation is beginning to emerge. In this regard, expression analysis performed on *ex vivo*-expanded human γδ T cells showed a remarkable Notch1 and Notch2 expression. Additionally, stimulation of γδ T cells with phosphoantigens triggers Notch activation (Gogoi et al., 2014), while inhibition of Notch signaling with GSI, leads to a reduction in TNFα, INFγ and IL17 production. Accordingly, specific knockdown of Notch1 and Notch2 inhibits γδ T cell antitumor cytotoxic potential, indicating that Notch signaling may regulate effector functions of γδ T cells (Gogoi et al., 2014).

In contrast to what occurs with αβ T cells, which acquire their functional competence at the periphery, functional maturation of γδ T cells occurs in the thymus, at least in mice (Narayan et al., 2012). Two main functional subsets of γδ T cells have been characterized so far, the IFNγ- and the IL17-secreting γδ T cells. In mice *ex vivo* isolated γδ T cells from the thymus produce IL-17 in response to PMA and ionomycin (Shibata et al., 2008), thus functional pre-programming occurs in the thymus. The developmental cues provided by the TME for specific functional differentiation of IL-17 or INFg γδ T cell producers are largely unknown, although intrathymic development of the naturally occurring IL-17–producing γδ T cells was shown to be dependent on the RORγt transcription factor (Ribot et al., 2009), and it is known that IL7 selectively activates the production of IL-17 by γδ T cells via STAT5 (Michel et al., 2012). Interestingly, the canonical Notch target Hes1 is specifically expressed in IL-17–producing γδ T cells and seems to be involved in their development, as RBPjκ directly interacts with the IL17 promoter (Mukherjee et al., 2009). Additionally, Dll4–expressing stromal cells support the development of IL-17–producing γδ T cells *in vitro*. All these evidences support a role for Notch signaling in the functional differentiation of γδ T cells (Shibata et al., 2011).

3.4. Role of Notch signaling in TEC development and homeostasis.

As discussed above, an essential part of TEC development depends on crosstalk signals delivered from developing thymocytes. Characterization and understanding of those signals is important to address thymic regeneration and thus improvement of the immune response in the elderly and in patients that undergo inmmunoablative treatments. However, little is known about the nature of those signals, although the Notch pathway could be involved, as it has been shown that TECs express Notch receptors (Felli et al., 1999) (Radtke et al., 2004). Interestingly, Notch signaling is involved in epithelial cell development in other organs like the skin and the gut (Nowell and Radtke, 2013). Additionally, recent *in vitro* studies in mice suggest that Notch signaling from thymocytes to TECs could induce TEC development at an early phase of thymic organogenesis (Masuda et al., 2009). Therefore, thymic crosstalk could include reciprocal Notch signaling between TECs and thymocytes, but evidence that this is actually the case is still scarce.

Likewise, little is known about the possible involvement, of Notch signaling in the natural process of age-related thymic atrophy, known as thymic involution (Haynes et al., 2000). This process results in a progressive decline in naive T cell generation, leading to a loss of peripheral T cell diversity. The involuted thymus suffers several molecular and histological changes (Fig. 13), including a dramatic decline of FoxN1 expression (Ortman et al., 2002). In humans, total thymocyte numbers decline after the first year of life (Bertho et al., 1997), although the more accused loss of thymocyte counts coincides with puberty and the release of sex steroids (Hince et al., 2008). This is accompanied by a decline in total thymus weigh, an increase in adipose tissue and a disorganization of the cortex and medulla. Besides, it is known that the size of Hassal's bodies and cysts increases with age (Steinmann et al., 1985), that there is higher frequency of "epithelial-free" areas (Aw et al., 2008), and that the PVS enlarges (Flores et al., 1999) (Haynes et al., 2000); but little is known about the molecular mechanisms underlying this processes. *In situ* quantification of epithelial markers revealed an age-dependent decline of cortical and medullary markers (such as keratin-8 and MHC-II) and IL-7 production (Alves et al., 2009) (Alves et al., 2010) (Aw and Palmer, 2011), together with an increase in Notch1 and Dll1 expression (Ortman et al., 2002) (Aw et al., 2009). In contrast, Dll4 is downregulated in cTECs in aged mice (Fiorini et al., 2008). Further evidence supporting a role for Notch in thymic involution comes from *in vivo* studies showing that activation of Notch signaling in TECs via Jag1 is associated with premature thymic atrophy (Beverly et al., 2006). All these data suggest that thymic involution encompasses changes in the TME that may affect Notch signaling in TECs. However no evidences of *in vivo* Notch activation in TECs have been provided to date.

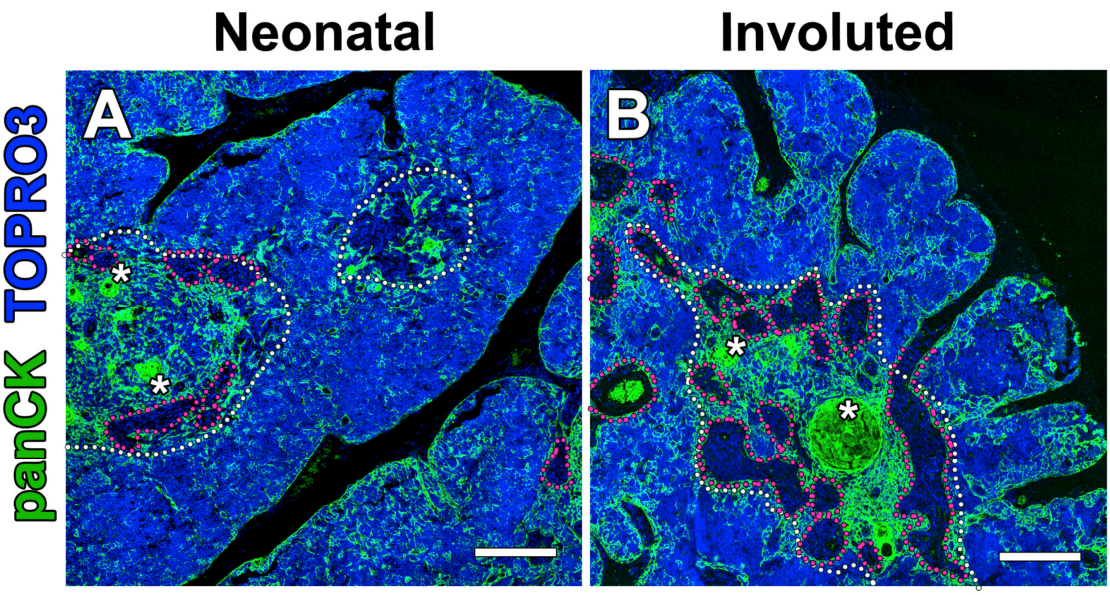
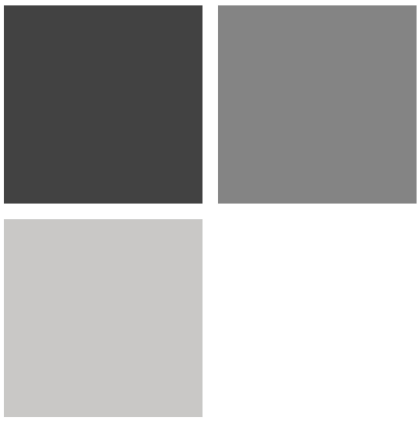


Figure 13. Histologic changes of the TME with thymic involution. A, Neonatal human thymus (17 months-old). B, Involuted human thymus (11 years-old). Age-related enlargement of Hassal's corpuscles, PVS and interlobular septae decrease the functional volume of the thymic parenchyma. *White-dotted line:* CMJ; *magenta-dotted line:* PVS; *asterisks:* Hassal's corpuscles. Scale bar: 100 um.



Specific Aims

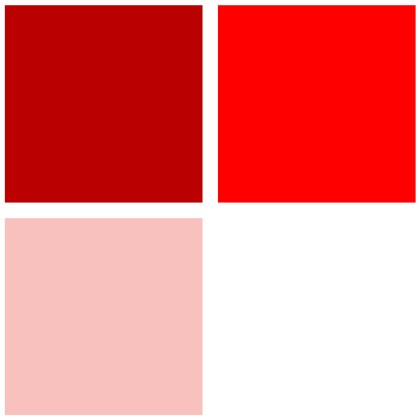
The general goal of this study has been to determine whether a differential distribution of Notch ligands and receptors in the human thymus could define discrete microenvironments with specific developmental functions, paying special attention to the critical check-point that controls the split of developing thymocytes into αβ and γδ T cells, and also to the physiological role of the Notch pathway in the biology of the thymic epithelium. To address this goal, we propose the following specific aims:

1. To characterize the regulation of Notch signaling activation and the expression of Notch ligands and receptors during thymus ontogeny and involution in human and mouse.

2. To investigate the contribution of different Notch ligands to the human αβ/γδ developmental branch point as well as to γδ T cell generation, expansion and functional maturation.

3. To assess the dynamics of γδ T cell development in the human thymus.

4. To study the role of Notch signaling in TEC biology using a conditional loss-of-function murine model.



Materials and methods

1. Tissue processing and immunohistochemistry.

1.1. Histology proceedings.

Experiments were performed in accordance with procedures approved by the Spanish Research Council Bioethics Committee. Human thymic biopsies were obtained from newborn to 15 years-old children undergoing cardiac surgery, after informed consent was provided in accordance with the Declaration of Helsinki. Murine tissue samples were obtained from 5 to 16 week-old C57BL/6 mice, and from 3 to 12 months-old C57BL/6 RBPjk-KO^{TEC} mice. Tissue samples were fixed over-night (O/N) at 4ºC in 4% paraformaldehyde/PBS (PFA, Sigma-Aldrich) and paraffin embedding was performed following a dehydration protocol using graded ethanol series, xylene washes and a final paraffin embedding step (Paraplast Plus, Sigma-Aldrich). Timing of each inclusion step was specifically optimized for human and murine thymic tissue. Formalin-fixed paraffin-embbed (FFPE) human fetal thymic samples were kindly provided by Dra. N. Torán (Anatomic Pathology Service, Vall d' Hebron Hospital, Barcelona, Spain). In parallel, some other human thymic fresh tissue samples were included in O.C.T compound (Tissue-Tek, Sakura), snap-frozen and stored at -70ºC. From both types of tissue-embed specimens, serial 8 um sections were obtained (Reichert-Jung Frigocut 2800N) and mounted on poly-lysine-coated slides (SuperFrost Ultra Plus, Thermo Scientific). Prior immunostaining, FFPE tissue slides were deparaffinised in xylene and ethanol-graded series. Next, tissue antigens were retrieved by boiling samples in a microwave oven for 10 to 15 minutes in sodium citrate (10mM, pH 6.0). Slides were allowed to cool down to room temperature (RT) and washed in distilled water and phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched using 1% H2O2 in 100% methanol (40 min, RT) followed by washes in PBS and PBS 0.3% Triton-X-100 (Sigma-Aldrich). For blocking unspecific antibody binding sites, samples were incubated 1 hour in blocking solution (3% BSA, 20mM MgCl2, 0.3% Tween-20, 5% FBS in PBS) in humidified chamber prior incubation with suitable primary antibodies.

In the case of frozen thymic tissue, air-dried tissue slides were fixed 10 minutes in cold acetone and washed in PBS. Then, tissue autofluorescence was quenched with 50mM NH_4Cl and saturated Sudan Black solution (Sigma-Aldrich). Endogenous peroxidase was quenched when necessary as previously described and, unspecific antibody binding sites were blocked with blocking solution without surfactant (3% BSA, 20mM $MgCl_2$, 5% FBS in PBS).

1.2. Immunofluorescent staining of Notch ligands, Notch receptors and Hes1.

Deparaffinised, rehydrated and properly blocked FFPE tissue slides were permeabilized 20 minutes in PBS 0.3% Triton-X-100, and incubated in humidified chamber

(O/N; 4ºC) in blocking solution containing the following primary antibodies: anti-human Dll1, anti-human Dll4, anti-human Jag1, anti-human Jag2, anti-human Notch1, anti-human cleaved Notch1, anti-human Notch3, anti-human Notch4 and anti-human Hes1 (see Table 1). Background and unspecific staining was determined by incubating with irrelevant antibodies, specie-specific normal serum and/or in the absence of primary antibody. Prior addition of secondary antibodies, tissue endogenous biotin was quenched by incubation in Avidin/Biotin blocking solutions (Vector Labs). For immunodetection of Dll4, Jag1 and Jag2, tissue slides were then incubated 1 hour at RT with HRP-coupled anti-rabbit IgG antibody (in 5% BSA in PBS, DAKO) and then, the signal was amplified by the Cyanine-3 Tyramide Signal Amplification kit (TSA; NEL 744, Perkin Elmer). TSA-Cy3 incubation time was 3.5 minutes. For Dll1, Notch1, Notch3, Notch4 and cleaved Notch1 (ICN1) immunodetection, a biotinylated anti-rabbit IgG antibody (Vector Labs) was added (5% BSA in PBS; 1 hour) prior signal amplification which comprised two steps. First, slides were incubated 1 hour with Avidin/Biotin-HRP complex (Elite Vectastain ABComplex kit, Vector Lab.) and then HRP was developed by adding TSA-Cy3 for 3.5 minutes. Finally, nuclei were stained with Topro-3 and slides mounted with Fluoromount-G (Southern Biotech.)

1.3. Immunofluorescent staining of thymic stromal cells.

For thymic stromal cell (TECs, myeloid cells, perivascular mesenchymal cells and endothelium) characterization, deparaffinised, rehydrated and properly blocked FFPE tissue slides were permeabilized 20 minutes in PBS 0.3% Triton-X-100, and incubated in humidified chamber (O/N; 4ºC) in blocking solution. For total TECs immunostaining we used a mouse anti-human pan-cytokerain (panCK) antibody mix or a rabbit anti-human cytokeratin 19 antibody. For myeloid cells immunostaining we used a mouse anti-human CD11c and. Finally, for mesenchymal and endothelial cells we used a mouse anti-human CD34 mAb (see Table 1). For CD34 immunodetection on intrathymic progenitors and, prior addition of secondary antibodies, tissue endogenous biotin was quenched by incubation in Avidin/Biotin blocking solutions (Vector Labs). Then, tissue slides were incubated one hour in humidified chamber (RT) with a biotinylated IgG anti-mouse antibody (5% BSA in PBS; Vector Laboratories) following 1 hour incubation with Avidin / Biotin-HRP Complex (Elite Vectastain ABComplex kit, Vector Labs). Next, ABC-amplified CD34 specific signal was developed by adding Alexa-488- or Alexa-555-conjugated Streptavidin for 20 minutes. For the detection of CD34 in mesenchymal areas (capsule, septae and PVS) no signal amplification was used, and primary antibody signal was developed directly with an Alexa-555 coupled goat anti-mouse antibody. CD11c, CK19 and panCK were developed without signal amplification by one hour incubation in humidified chamber (RT) with an Alexa-488-

coupled goat anti-mouse antibody or an Alexa-488-coupled goat anti-rabbit antibody. Background and unspecific staining was determined by incubating with irrelevant antibodies, specie-specific normal serum and/or in the absence of primary antibody. Finally, nuclei were stained with Topro-3 and slides were mounted with Fluoromount-G (Southern Biotech.).

1.4. Immunofluorescent staining of TCRγδ cell populations.

In vivo detection of TCRγδ cells in the human thymus was addressed in frozen acetone-fixed thymic tissue. Briefly, H_2O_2-quenched and blocked tissue slides were incubated with the following primary antibodies: mouse anti-human TCRγδ, mouse anti-human Vδ1, mouse anti-human Vδ2, mouse anti-human intracellular TCRβ (kindly provided by M. Brenner, Brigham and Women's Hospital, Boston, MA) and mouse anti-human CD1a (see Table 1). Human Vδ1 and Vδ2 γδ T cells show two distinct molecular configuration of the TCR. In Vδ2 γδ T cells TCRδ and TCRγ chains are disulfide-linked, while Vd1 TCRd and TCRgd chains are not disulfide linked (Brenner et al., 1987). This molecular characteristic allow to detect these two γδ T cell subpopulations with different monoclonal antibodies (Bottino et al., 1988) (Grossi et al., 1992) that we have used to locate Vδ1 and Vδ2 gd T cells *in vivo* in the human thymus. Background and unspecific staining was determined by incubating with irrelevant antibodies, specie-specific normal serum and/or in the absence of primary antibody. For TCRδ, Vδ1, Vδ2 and TCRβ detection an Alexa-488- or Alexa-555-coupled anti-mouse IgG1 secondary antibody was added (5% BSA in PBS; Vector Laboratories, 1 hour) depending on the combination with the stromal marker CK19 (see above) or CD1a. For CD1a immunodetection an Alexa-555-coupled anti-mouse IgG2a secondary antibody was used. For nuclei were stained with Topro-3 and slides mounted with Fluoromount-G (Southern Biotech.).

1.5. Haematoxilin/eosin and β-galactosidase (LacZ) stainings.

FFPE tissue slides of postnatal (< 17 months-old) and involuted (> 6 years-old) human thymic tissue were stained with haematoxilyn/eosin for tissue morphology observations. Briefly, deparafinized tissue slides were incubated for 3 minutes in Harry's haematoxilyn (Sigma), washed and quickly differentiated (10 to 15 sec) in acid alcohol solution (0,5% HCl; 70% ethanol). Next, they were incubated 9 minutes in 0,5% (w/v) Eosin solution (Sigma) and sequentially dehydrated in graded ethanol series. Finally, tissue slides were briefly incubated in xylene and mounted with Entellan mounting medium (Merck, Millipore).

For β-galactosidase staining, intact thymic lobes from 3 days-old and 8 weeks-old C57BL/6J Rosa26-LacZ$^{flox/flox}$ and B6D2F1/J FoxN1-Cre-Rosa26-LacZ$^{flox/flox}$ mice were fixed in 0,125% glutaraldehyde/PBS solution for 30 min at RT. Next, thymic lobes were washed in wash buffer (0,02% Nonidet-P40; 0,11% sodium deoxycholate and MgCl2 2mM; in phosphate buffer 0,1M; pH 7,3) and stained O/N RT in X-gal staining solution (wash buffer supplemented with potassium ferricyanide 5mM, potassium ferrocyanide 5mM and 1mg/ml of X-gal resuspended in N,N-dimethyformamide). The cleavage of X-gal (5-bromo-4-chloro-3-indolyl-b-galactopyranoside) by b-galactosidase results in a dark blue precipitate. After X-gal staining, thymic lobes were washed in wash buffer, fixed in 4% PFA/PBS (Sigma-Aldrich) and paraffin-embed as described previously. Serial 8 um sections were mounted on poly-lysine-coated slides (SuperFrost Ultra Plus, Thermo Scientific) and deparaffinised as specified earlier. Cell nuclei were stained with Nuclear Fast Red (Vector Labs), sequentially dehydrated in graded ethanol series and xylene, and mounted with Entellan mounting medium (Merck, Millipore).

1.6. Table of antibodies used in immunohistochemical analysis.

Specificity	Reactivity	Clone	Isotype	Origin	Conjugate
Anti-mouse IgG1	M	Polyclonal	Goat	Thermo	Alexa-488
Anti-mouse IgG2a	M	Polyclonal	Donkey	Thermo	Alexa-555
Anti-mouse IgG	M	Polyclonal	Goat	Vector Lab.	Biotin
Anti-Rabbit IgG	Rbb	Polyclonal	Goat	DAKO	HRP
Anti-Rabbit IgG	Rbb	Polyclonal	Goat	Vector Lab.	Biotin
Anti-Rabbit IgG	Rbb	Polyclonal	Donkey	Thermo	Alexa-488
CD11c	H	5D11	Mouse IgG2a	Novocastra	Purified
CD1a	H	Na1/34	Mouse IgG1	AbcSerotec	Purified
CD34	H	My10	Mouse IgG1	J.H. Shaper	Purified
Cleaved Notch1	H, M	D3B8	Rabbit IgG	Cell Sign.	Purified
Dll1	H, R	Polyclonal	Rabbit IgG	Abcam	Purified
Dll4 (H-70)	H, M, R	Polyclonal	Rabbit	Sta. Cruz	Purified
Hes1	H, M	D6P2U	Rabbit IgG	Cell Sign.	Purified
Jag1	H, M	28H8	Rabbit IgG	Cell Sign.	Purified
Jag2 (2205)	H	C83A8	Rabbit IgG	Cell Sign.	Purified
Jag2 (2210)	H, M	C23D2	Rabbit IgG	Cell Sign	Purified
Cytokeratin 19	H	Polyclonal	Rabbit	Sigma	Purified
Notch1 (C-20)	H	Polyclonal	Goat	Sta. Cruz	Purified
Notch3	H	Polyclonal	Rabbit IgG	Abcam	Purified

Notch4	H	Polyclonal	Rabbit IgG	Abcam	Purified
Pan-cytokeratin	H, M, Rbb	Polyclonal mixture	Mouse IgG1 and IgG2a	SIGMA	Purified
TCR Vδ2	Human	BB3	Mouse IgG1	L. Moretta	Ascytes
TCR Vδ1Jδ1	H	TSC1 (TS-1)	Mouse IgG1	Thermo Sc.	Purified
TCRβ	Human	bF1	Mouse IgG1	M. Brenner	Purified
TCRγδ	H	B1.1	Mouse IgG1	eBioscience	Purified
TCRγδ	Human	5A6.E9	Mouse IgG1	Invitrogen	Purified

Table 1. Antibodies used in immunohistochemistry. Legend: H: human; M: mouse; R: rat; Rbb: rabbit.

2. Acquisition, analysis and statistics of confocal microscopy images.

Images were acquired using an LSM510 laser scan confocal microscope (Zeiss) coupled to an Axio Imager.Z1 or an Axiovert 200 (Zeiss) microscope using the following magnifications (Zeiss): 10× Plan-Neofluar (numeric aperture [NA] 0.3), 25× Plan-Neofluar (oil [NA] 0.8), 40× Plan-Neofluar (oil [NA] 1.3) and 63x Plan-Apochromat (oil [NA] 1.4). Images were obtained at 1024px x 1024px resolution and 8-bit colour depth for descriptive purposes and at 12-bit for quantifications. Images were processed using ImageJ and Adobe Photoshop CS4 software (brightness and contrast were adjusted equally in samples and controls). Co-expression, Mean Fluorescence Intensity (MFI) analyses and histomorphometric measurements were performed in ImageJ by definition and comparison of ROIs (region of interest) created by threshold of intensity of fluorescence (image thresholding) by applying the Otsu algorithm (Otsu, 1979). M1 and M2 Mander's coefficients were obtained by applying the Intensity Correlation Analysis (ICA) plugin *(http://www.uhnresearch.ca/facilities/wcif/imagej/colour_analysis.htm)* to thresholded images. When comparing MFI in TECs from different anatomic regions of the same sample (25x magnification images), equivalent square ROIs (100µm x 100µm) were defined on each region on which application of intensity threshold (Otsu, 1979) allowed us to create a final specific ROI in TECs. Cell counting analyses were performed in Adobe Photoshop CS4 (Extended) by using the "Count" tool. Data are presented as mean +/- SEM and statistical analyses were performed using Graph Pad software. Statistical significance was determined with the unpaired 2-tailed Student *t* test and one-way ANOVA, with the α level set at 0.05.

3. Immunomagnetic isolation of human primary thymocyte subsets.

Normal human postnatal thymocytes were isolated by mechanical disruption of thymus fragments, removed during corrective cardiac surgery of patients aged from 3 days up to 4 years, after informed consent was provided in accordance with the Declaration of

Helsinki. Experiments were performed in accordance with approved guidelines established by the Research Ethics Board of the Spanish Research Council. A thymocyte cell suspension was obtained after Ficoll-Hypaque (Lymphoprep™; ATOM) centrifugation of the total cell suspension resulting from mechanical disruption of the thymic tissue. For CD34+ intrathymic progenitors isolation, thymocyte cell suspension was subsequently enriched in non-T cells by sheep erithrocyte rosseting, as previously described (Martin-Gayo et al., 2010). Next, CD34+ cells were selected from the resulting cell fraction using magnetic-activated cell sorting with CD34+ MicroBeads (Dynal, CD34 Progenitor Cell selection System, Invitrogen). Two different populations of intrathymic progenitors, CD34+ cells (DN2) and $CD34^{hi}$ cells (ETPs), were obtained from the CD34-labeled fraction by sequential incubations with an anti-CD34 competitor antibody (DETACHaBEAD, Dynal, Invitrogen) at 25ºC and 37ºC, respectively. Additionally, selected $CD34^{hi}$ and CD34+ populations were depleted from CD1a+ cells by the incubation with anti-CD1a MicroBeads (MACS, Miltenyi Biotec) and negative selection using an AutoMACS magnetic cell sorter (Miltenyi Biotec), obtaining a CD34+ CD1a- population (DN2 progenitors). The resulted positive fraction was depleted from CD4+ cells by incubation with anti-CD4 Microbeads (MACS, Miltenyi Biotec) obtaining a CD34 CD1a+ population (proT cells).

For total human intrathymic TCRγδ cell isolation, the thymocyte cell suspension obtained after Ficoll-Hypaque (Lymphoprep™; ATOM) centrifugation was left O/N at 4ºC in RPMI supplemented with 10% of FBS, and then subjected to a second Ficoll-Hypaque centrifugation to remove apoptotic thymocytes. The resultant fraction was incubated with Hapten-labelled anti-human TCRγδ mAb and FITC-labelled anti-Hapten MicroBeads (Human TCRγδ+ Isolation kit, MACS, Miltenyi Biotec), and isolated by manual magnetic cell sorting using positive selection columns (MACS LS Column, Miltenyi Biotec). For the specific isolation of CD1a+ immature γδ T cells, the thymocyte cell suspension obtained from the second Ficoll-Hypaque centrifugation was fractionated by centrifugation on Percoll density gradients (GE Healthcare). Big size thymocytes recovered from the 1.068 g/ml density layer (F50) were then incubated with PE-labelled anti-human CD1a mAb (Coulter) and anti-PE MicroBead Multi-Sort Kit (MACS, Miltenyi Biotec). Then, $CD1a^{hi}$ cell were isolated by manual magnetic cell sorting using positive selection columns (MACS LS Collumn, Miltenyi Biotec). Next, TCRγδ cells were selected from the $CD1a^{hi}$ thymocyte population (purity 100%) by incubation with Hapten-labelled anti-human TCRγδ mAb and FITC-labelled anti-Hapten MicroBeads (Human TCRγδ+ Isolation kit, MACS, Miltenyi Biotec). Finally, CD1ahi TCRγδ-labelled cells were isolated by manual magnetic cell sorting using positive selection columns (MACS LS Collumn, Miltenyi Biotec) (purity $\geq$ 90%).

4. Isolation of peripheral blood lymphocytes (PBLs).

Human peripheral blood samples (buffy coat) obtained from healthy individuals were used to isolate PBLs, after informed consent was provided in accordance with the Declaration of Helsinki. Briefly, total mononuclear blood cells were obtained by Ficoll-Hypaque (Lymphoprep™; ATOM) density gradient centrifugation. The resulted fraction of mononuclear cells was incubated with erythrocyte lysis buffer (9 vol. NH_4Cl 0,83%; 1 vol. Tris HCl 2%) to discard erythrocyte remnants. Next, mononuclear blood cell suspension was enriched in lymphoid cells (T and NK cells mainly; purity > 90%) by depletion of myeloid lineage cells using lineage specific antibodies followed by negative selection using an AutoMACS magnetic cell sorter (Miltenyi Biotec).

5. Flow cytometry.

5.1. Cell membrane staining.

Single cell suspensions of lymphocytes from murine thymus and spleen, were prepared by standard procedures**.** Additionally, the expression of surface markers on human primary and *in vitro* cultured thymocytes was determined by staining (for three to six colors) of 2 x 10^4 cells per condition in ice-cold FACS buffer (PBS, 1% BSA, 3mM EDTA). The direct fluorochrome- or biotin-labelled mAbs used are detailed in Table 2, as well as, second-step reagents including PE-conjugated goat anti-mouse IgGs or PE- or APC-conjugated streptavidine. Data were collected on 1 to 5 x 10^3 viable cells as determined by electronic gating on forward and side scatter light parameters. Isotype-matched irrelevant antibodies were used as negative controls to define background fluorescence. Three-colour flow cytometry acquisition was performed using a FACSCalibur cytometer and the CellQuest software (BD Biosciences), up to six-colour flow cytometer acquisition was performed in a FACSCanto II and FACSDiva software. FACS Flow cytometric data analysis was performed using FlowJo vX (LLC software).

5.2. Intracelullar stainings.

Intracelullar TCR-β expression on *ex-vivo* or *in vitro* developing thymocytes was assessed using the CytoFix-CytoPerm Permeabilization Kit (BD Biosciences) following manufacturer's intructions. Briefly, 5 x 10^4 cells were fixed and incubated with anti-human βF1 mAb (provided by M. Brenner, Brigham and Women's Hospital, Boston, MA) in CytoPerm solution. Then, PE-labelled anti-mouse IgG1 secondary antibody was added to the

cells. A PE-labelled irrelevant isotype-matched antibody was used as control. Next, and after proper blocking in normal mouse serum, mAbs for staining of other markers were added.

IL-17 and INFγ cytokine expression on *ex-vivo* or *in vitro* generated γδ T cells and human PBLs was assessed in activated cells (see 11.3) by fixation of previously membrane-stained 5 x 10^4 cells in 2% PFA/PBS for 10 minutes at RT. After proper wash, PE-labelled anti-human IL-17 or anti-human IFNγ mAbs were added to the cells in permeabilizing solution (FACS Buffer; 0,5% Saponin/PBS pH 7,4) and incubated for 30 minutes at RT. Then, cells were washed in permeabilizing solution and re-suspended in FACS buffer.

5.3. Table: Summary of antibodies used in flow cytometry.

Specificity	Reactivity	Clone	Isotype	Origin	Conjugate
CD1a	Human	SFCI19Thy1A8	Mouse IgG1	B. Coulter	PE
CD1a	Human	Na1/34	Mouse IgG1	AbcSerotec	Biotin
CD3	Human	B-B11	Mouse IgG1	Diaclone	FITC
CD3	Human	UCHT1	Mouse IgG1	BD	PE, APC
CD3	Human	S4.1	Mouse IgG2a	Life Technol.	TC
CD4	Human	13B8.2	Mouse IgG1	B. Coulter	FITC, TC
CD4	Human	SFCI12T4D11	Mouse IgG1	B. Coulter	PE
CD5	Human	L17F12	Mouse IgG2a	BD	FITC
CD7	Human	CD4-6B7	Mouse IgG2a	Life Technol.	PE-Cy5
CD8a	Human	G42-8	Mouse IgG2a	BD	FITC
CD8a	Human	3B5	Mouse IgG2a	Life Technol.	PE
CD8a	Human	RPA-T8	Mouse IgG1	BD	PE-Cy7
CD8b	Human	2ST8.5H7	Mouse IgG2	B. Coulter	PE
CD27	Human	M-T271	Mouse IgG1	BD	FITC, PE
CD33	Human	D3HL60.251	Mouse IgG1	B. Coulter	PE-Cy5
CD34	Human	581	Mouse IgG1	B. Coulter	FITC, PE-Cy5
CD44	Human	G44-26	Mouse IgG2b	B. Coulter	FITC
CD56	Human	MEM-188	Mouse IgG2a	Caltag	PE
CD69	Human	CH/4	Mouse IgG2a	Life Technol.	PE
CD127	Human	R34.34	Mouse IgG1	B. Coulter	PE
IFNγ	Human	B27	Mouse IgG1	BD	PE
IL-17A	Human	eBio64DEC17	Mouse IgG1	eBioscience	FITC
Mouse IgG1	Mouse	Polyclonal	Goat IgG1	Southern Bio	FITC, PE-Cy5
Mouse IgG1 Isotype Control	-	MOPC-21	Mouse IgG1	BD	FITC, PE, PE-Cy5, APC

Mouse IgG2a Isotype Control	-	G155-178	Mouse IgG2a	BD	PE
Notch1	Human	MHN-519	Mouse IgG1	Biolegend	PE
Notch3	Human	MHN3-21	Mouse IgG1	Biolegend	PE
Biotin	-	-	Streptavidin	Biolegend	APC
TCRαβ	Human	IP26A	Mouse IgG1	B. Coulter	PE-Cy5
TCRγδ	Human	IMMU510	Mouse IgG1	B. Coulter	PE
TCRγδ	Human	5A6.E9	Mouse IgG1	Life Technol.	Pe-Cy5
TCRγδ	Human	B1	Mouse IgG1	BD	APC
TCR Vδ1	Human	TS8.2	Mouse IgG1	Thermo Sc.	FITC
TCR Vδ2	Human	B6	Mouse IgG1	Biolegend	PerCP
TCR Vδ1	Human	TSC1 (TS-1)	Mouse IgG1	Thermo Sc.	Purified
TCR Vδ2	Human	BB3	Mouse IgG1	L. Moretta	Ascytes
TCRβ	Human	bF1	Mouse IgG1	M. Brenner	Purified

Table 2. Antibodies used in flow cytometry.

6. RNA Isolation.

Total RNA was obtained, by using TRIzol reagent (Invitrogen Life Technologies) according to manufacturer's instructions, from the following sets of human samples: CD34+ CD1a- lymphoid progenitors (LP), total TCRγδ+ cells, CD34+ CD1a+ pro-T cells generated *in vitro* under different culture conditions (GFP, Dll4, Jag1 and Jag2) and HeLa cell line. RNA concentration and purity was determined by 260/280 (< 1.8) and 260/230 (< 1.8) absorbance ratios (Nanodrop ND-100, Thermo Scientifc).

7. Polimerase chain reaction (PCR).

7.1. Reverse transcriptase PCR (RT-PCR).

RT-PCR assay was performed for the amplification of the human Jagged2 c-terminal region. To prepare total RNA for reverse transcription of Jag2 DNA, HeLa cells (ATCC) were lysed in TRIzol Reagent (Invitrogen) and extracted according manufacturer's instructions. Next, cDNA was reverse transcribed using oligo $(dT)_{12\text{-}18}$ primers (Invitrogen) and Expand Reverse Transcriptase (Roche). WT human JAG2 c-term (1.8 Kb fragment containing the TAG stop codon) was PCR amplified from total HeLa cDNA by Jag2 c-term specific primers, containing hanging NotI restriction sites. The upstream primer was the following: agccGCGGCCGCtgctacga. The downstream primer was the following:

atatGCGGCCGCctactccttgccggcgtagcgg (NotI restriction sites are given in capital letter). PCR reaction was performed using X Pfu polymerase (Roche). Samples were denatured at 95ºC for 2 minutes and amplified at 95ºC for 30 seconds, 65ºC for 30 seconds and 72ºC for 2 minutes followed by a final extension at 72ºC for 10 minutes. cDNA integrity was assessed by parallel amplification of β-actin (primers). Amplified PCR products were subjected to 1 % agarose (Promega) electrophoresis and visualized with ethidium bromide.

7.2. Cuantitative PCR (qPCR).

Real-time PCR quantification of complementary DNA synthesized from TRIzol-extracted (Invitrogen) total RNA using oligo $(dT)_{12-18}$ primers (Invitrogen) was performed using TaqMan Gene Expression Assays (Applied Biosystems), according to the manufacturer's instructions, in a ABI PRISM 7900 HT Sequence Detection system (Applied Biosystems). The TaqMan probes used are detailed in the following table.

Target mRNA	TaqMan reference	Target mRNA	TaqMan Reference
TOX2	Hs00262775_m1	LEF1	Hs01547250_m1
ITGAE	Hs01025372_m1	THEMIS	Hs01041269_m1
GATA3	Hs00231122_m1	FOXO1	Hs01054576_m1
SOX13	Hs00232193_m1	BLK	Hs01017482_m1
ETV5	Hs00927557_m1	HES1	Hs00172878_m1
RUNX3	Hs00231709_m1	GAPDH	Hs0258991_g1

Table 3: TaqMan probes used in qPCR assays.

8. DNA cloning into retroviral and expression vectors.

The pLZRS-polylinker-IRES-enhancedGFP retroviral constructs encoding either the full-length human Dll1 or Jag1, and the GFP empty vector were kindly provided by Dr L. Parreira (Instituto de Histologia e Embriologia, Lisboa, Portugal). Human Dll4 full-length cDNA in the pcDNA3.1/myc-His expression vector, was a generous gift by Dr G. Tosato (National Institutes of Health, Bethesda, MD). Dll4 cDNA was excised from the pcDNA3.1 expression vector and cloned into the BamHI and EcoRI restriction sites of the pLZRS-linker-IRES-eGFP retroviral construct. Restriction and sequence analysis were performed to confirm proper ligation of the human Dll4 open reading frame into the pLZRS-IRES-eGFP vector. A myc-tagged human JAG2 cDNA cloned in the pcDNA3 expression vector was kindly provided by Dr P. Menendez (Andalusian Stem Cell Bank, Spain). This JAG2 construct was

lacking the last 20 aminoacids (C-term) of the JAG2 protein, carrying instead a tandem of 3 myc-tag copies. As endocytosis of C-terminal region of Notch ligands is necessary for proper Notch signalling, WT human Jag2 C-term cDNA (1,8 Kb fragment containing the TAG stop codon) was PCR amplified from total RNA isolated from HeLa cells as described above (see section 7.1). Finally, for Jag2 full-length composite, Jag2-myc-tagged cDNA was excised from the pcDNA3/myc-His using HindIII and XbaI restriction sites and cloned into a pBluescript II SK- intermediate vector where the fragment of 1,8 Kb containing the C-terminal myc-tag tandem was removed and discarded by enzimatic restriction with NotI enzyme. Next, the remaining N-terminal portion of JAG2 (2,1 Kb fragment containing the ATG initiation codon) was also excised using the EcoRI site available at the pBluescript II SK- cloning cassette. Finally, the N-terminal 2,1kb JAG2 fragment (EcoR-NotI) and the WT PCR-amplified JAG2 c-term 1,8Kb fragment (NotI-NotI) were sequentially ligated into the final pLZRS-IRES-GFP retroviral vector. Restriction and sequence analysis were performed to confirm proper amplification and ligation in correct orientation of both JAG2 fragments in the LZRS-IRES-eGFP vector.

9. Generation of retroviral particles and retroviral transduction of the OP9 stromal cell line.

Dll1, Dll4, Jag1, Jag2 and GFP retroviral constructs were used for lipofection (Fugene6, Roche) of the packaging 293T Phoenix-Amphotropic cell line (kindly provided by G. Nolan, Stanford University, Stanford, CA). 48 hours after transfection, transfected 293T Phoenix-Ampho cells were selected by addition of 2,5 µg/ml of puromycin (Sigma). Two weeks after transfection, puromycin-selected cells were plated confluent on p100 plates (Falcon) of DMEM medium supplemented with 10% fetal calf serum and 1% HEPES (Gibco, Invitrogen) without puromycin, from which we obtained high titres of empty (control GFP only) or Dll1, Dll4, Jag1 and Jag2 recombinant retrovirus. The following day retroviral supernatants were harvested and used for transduction of the OP9 stromal cell line (ATCC). OP9 cells were seeded semi-confluent onto p24 well plates and transduction was performed by centrifugation at 1800 rpm in the presence or 8 µg/ml of polybrene. 48h post-transduction OP9 cells were analyzed for GFP expression by flow cytometry and highly transduced cells (between 10^3 – 10^4 log) were sorted until 100% of the cells expressed homogeneous levels of GFP.

10. Western blotting.

Ectopical overexpression of Notch ligand on the OP9-engineered cell lines was confirmed by Western blot. OP9 cells were lysed in RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 0.5% Sodium deoxycholate, 1% NP-40, 0.1% SDS) plus a protease inhibitor cocktail containing sodium fluoride, sodium orthovanadate, aprotinin, pepstatin A and PMSF (all from Sigma). Protein quantification was performed using BCA assay (MicroBCA^{TM}, Pierce) and equal amounts of total protein from OP9-GFP, OP9-Dll1, OP9-Dll4, OP9-Jag1 and OP9-Jag2 were elecrophoretically separated and transferred to PVD blotting membranes (Millipore). Membranes were blocked 1 hour in 5% non-fat milk TBS-T buffer (TBS plus 0.05% Tween-20, Sigma-Aldrich) prior incubation with the following primary antibodies: rabbit anti-human Dll1 (Abcam), rabbit anti-human Dll4 (Abcam), rabbit anti-human Jag1 (Cell Signaling) and rabbit anti-human Jag2 (Cell Signaling). Mouse anti–α–tubulin mAb (Sigma) was used as loading control. Signal was developed with the appropriate secondary Abs conjugated to HRP (Jackson Laboratories) in conjunction with a luminescent substrate (ECL, Roche).

11. Cell culture.

11.1. Co-culture of human intrathymic progenitors and intrathymic TCRγδ cells.

In vitro differentiation of αβ and γδ T cells from DN2-like lymphoid human CD34+ intrathymic progenitors was performed as previously described using the OP9 system (see Introduction 2.2). Briefly, engineered Notch ligand^{hi}-expressing OP9 cells were seeded semi-confluent on p24 well plates (Falcon) and co-cultured during 31 days with DN2-like CD34+ CD1a- intrathymic progenitor cells in minimum essential medium (α-MEM, Invitrogen) supplemented with 20% fetal calf serum (Invitrogen), 200IU/ml of human recombinant Interleukin-7 (hrIL-7, PreproTech) and 100IU/ml of Flt3L (NIBSC) at 37°C, 5% CO_2. Co-cultures were initiated with around 1 x 10^5 cells per well and were harvested by forceful pipetting every 3 or 4 days for phenotype analysis. Seeding to a fresh OP9-seeded well was in all cases preceded by filtration of the total cell suspension through a 45µm filter, in order to separate developing thymocytes from stromal OP9 cells. In the case of priming short co-cultures for qPCR analysis of thymocyte populations, lymphoid progenitors were co-cultured for 6 days in the same conditions. Then, resulting proT cells were separated from the stroma by filtering as early mentioned and stroma remnants were discarded by plate adhesion during

20 minutes at 37ºC, so no RNA from OP9 stromal cells was in any case contaminating qPCR analyses.

Ex-vivo isolated CD1a+ γδ T cells were cultured at 5 x 10^5 cells/ml at 37°C, 5% CO_2 in p24-well plates seeded with a semi-confluent monolayer of either Dll4-, Jag1-, Jag2- or GFP empty vector- expressing OP9 cells in α-MEM media (Invitrogen) supplemented with 20% FBS (Invitrogen), hrFlt3L (PreproTech) and 200IU/ml of hrIL-7 (NIBSC). Co-cultures were initiated with around 5 x 10^5 cells per well and harvested by forceful pipetting every 3 or 4 days for phenotypic analysis.

11.2. *In vitro* activation of T cells.

Ex vivo isolated peripheral blood lymphocytes or TCRγδ cells and *in vitro* generated αβ and γδ T cells were activated in vitro by culturing them 36 hours onto p46-well plates coated with 10μg/ml of anti-CD3-coated (OKT3, purified) and, in the presence of hrIL-2 (Peprotech). As *in vitro* activation induces CD3 (and thus TCR) downregulation, cells were passed to a new plate without anti-CD3 and cultured for further 5 to 6 days to recover TCR expression. Then cells were treated with Brefeldin A (Sigma) and stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma) and ionophore prior FACS analysis.

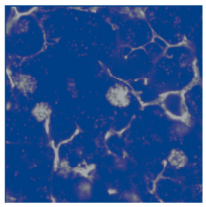
12. Mice.

Mice were kept under specific pathogen-free conditions and used according to institutional regulations. C57BL/6J WT mice were obtained in-house from the departmental breeding facility, whereas B6D2F1/J FoxN1-Cre mice were obtained from Dr. George Hollander (University of Basel) (Zuklys et al., 2009). C57BL/6J $RBPjk^{flox/flox}$ mice (Oka et al., 1995) and C56BL/6J Rosa26-$LacZ^{flox/flox}$ were obtained from Dr. Jose Luis de la Pompa (CNIC, Madrid), respectively. Mice genotyping was performed by PCR analysis of genomic DNA obtained by proteinase K (Sigma) digestion of 3 weeks-old mouse ear discs tissue. Primers used to detect Cre recombinase transgene were P1, 5'-TCT GAT GAA GTC AGG AAG AAC C-3', and P2, 5'-GAG ATG TCC TTC ACT CTG ATT C-3', which generates a fragment of about 500bp long. PCR were performed for 34 cycles of 94ºC (30 sec), 58ºC (40 sec), and 72ºC (1 min). For the detection of the $RBPjk^{flox/flox}$ alleles the following primers were used: P3, 5'-ACC AGA ATC TGT TTG TTA TTT GCA TTA CTG-3', and P4, 5'-ATG TAC ATT TTG TAC TCA CAG AGA TGG ATG-3', which generates a product of 430bp long for the $RBPjk^{flox/flox}$ allele and 274bp long for the WT allele. For specific detection of

RBPjκ deletion, the primer P5, 5'-TAA TGC ACA CAA GCA TTG TCT GAG TTC-3' was used in combination with primer P3 which yields no product in a non-deleted allele or a 640bp long product when the RBPjk gene has been successfully deleted. In this case, PCR was performed for 40 cycles of 94ºC (30 sec), 60ºC (1 min) and 72ºC (1 min). PCR products were subjected to agarose electrophoresis and visualized with ethidium bromide.

13. Statistical analysis.

Data are presented as mean +/- SEM and statistical analyses were performed using Graph Pad software. Statistical significance was determined with the unpaired 2-tailed Student *t* test and one-way ANOVA, with the α level set at 0.05.



Results

I. *IN VIVO* CHARACTERIZATION OF INTRATHYMIC NICHES DEFINED BY DIFFERENTIAL EXPRESSION OF NOTCH LIGANDS AND NOTCH RECEPTORS.

1.1. Dll1, Dll4, Jag1 and Jag2 Notch ligands are differentially expressed in the thymus, thus defining specific Notch signaling niches.

As described in detail in the Introduction, Notch signaling is a key player in T cell development. However, little is known about which Notch ligands or receptors are expressed *in vivo* in the thymus, and how they are distributed and regulated in the different thymic anatomic regions. This information is particularly scarce in the human thymus.

As T cell development is a step-wise process that occurs at discrete regions of the thymus, differential patterns of Notch ligand expression in those regions may be essential for shaping T cell developmental dynamics. Aware of this concern, our study on the characterization of intrathymic Notch ligand and receptor expression will focus on specific thymic anatomic regions, in order to obtain a better picture of each specific niche. Therefore, we will pursue the analysis of the spatio-temporal regulation of Notch ligands and Notch receptors expression in the human thymus, paying also attention to the mouse thymus for comparative purposes.

1.1.1. Notch ligands expression at the cortex and subcapsular region.

It has been reported that Delta family ligands are expressed *in vivo* in the cortex of the murine thymus (Schmitt et al., 2004) (Koch et al., 2008) (Hozumi et al., 2008). Our results confirmed Dll1 and Dll4 expression on murine cTECs; but, additionally, we found that rare non-epithelial cells in the cortex express both Delta ligands (Fig. 14B, C). When expression of Dll1 and Dll4 was analysed in human postnatal thymus samples (< 17 months-old), we found that Dll1 expression is quite conserved between mice and humans, as it is confined mostly to subcapsular cTECs (Fig. 15A) and expressed in low levels by IC cTECs and some thymocytes (Fig. 15B; 19A). But, in contrast to mouse, human cTECs do not express Dll4. Rather, only scarce subcapsular cTECs, together with few scattered hematopoietic cells and capillary endothelial cells express detectable levels of Dll4 in the human thymus cortex (Fig. 15E; 19A). This finding demonstrates that Dll4 expression is significantly different in the human and murine postnatal thymus.

Regarding the Jagged ligand family, it is known that Jag1 is completely absent from the cortex in the murine thymus (Lehar et al., 2005) and we found that this feature is conserved

in humans. Indeed, no single cell was found in our study to express Jag1 neither in the murine (Fig. 16A, B), nor the human thymus cortex (Fig. 17A, B; Fig 19A). But intrathymic Jag2 expression has been poorly addressed, as only analyses of Jag2 mRNA distribution in mouse thymus (Heinzel et al., 2007) (Mohtashami et al., 2010) and functional analyses of Jag2 KO mice (Jiang et al., 1998) have provided just a hint about the spatial regulation of this ligand in the intrathymic T cell niche. In this regard, we have found that Jag2 expression is prominent in the cortex, where it is exclusively expressed by cTECs both in mice (Fig. 16E) and humans (Fig. 17E; 19A). Despite both species share expression of Jag2 throughout the cortex, we found a specie-specific regulation at particular cortical niches: in mice, Jag2 expression is confined to cTECs at the SCC (Fig. 16A, E), while, in humans, Jag2 is broadly expressed at the cortex but specifically enriched in cTECs at the CMJ (Fig. 17D; see 1.1.2. below). This differential enrichment at either the SCC or the CMJ is another significant difference between the murine and human cortical niche.

1.1.2. Notch ligands expression at the CMJ.

The CMJ is a complex transitional region between the thymic cortex and the medulla, and we consider it as the narrow part of the inner cortex that it is in close proximity to the medulla. Epithelial cells at the CMJ are cTECs and, accordingly, Dll1 is fairly expressed at this location in both human and murine thyme (Fig. 14A, 15A). In contrast to Dll1, Dll4 is absent from cTECs at the CMJ, as it is from the rest of the cortex in the human thymus (Fig. 15A), but not in the murine thymus, where Dll4 is highly abundant at the CMJ (Fig. 14A).

Definitely, the most prominent ligand expressed at the CMJ in the human thymus is Jag2 (Fig. 17D). As mentioned previously, Jag2 expression is maximal at, and exclusive of, cTECs at the CMJ. In order to formally quantify this observation and, with the aid of the pan-cytokeratin epithelial marker (panCK), cTECs were selected in regions of interest (ROIs) by fluorescence intensity thresholding (Otsu, 1979). Several ROIs from cortex (COR; $N = 45$) and CMJ ($N = 45$) were defined from $N \geq 10$ independent images of Jag2-stained tissue (25x) per thymic sample ($N = 3$) (Fig. 18B). Then, cortical and corticomedulary ROIs were used to measure Jag2 mean fluorescent intensity (MFI) specifically on TECs (Fig 18C, D). Total Jag2 MFI values of cTECs from cortical regions (COR) and from the CMJ were statistically compared, obtaining a highly significant difference between both intrathymic niches (Fig. 18E). Collectively, these findings indicate that Notch ligand expression in cTECs, and particularly Jag2 expression, is differentially regulated at specific intrathymic niches.

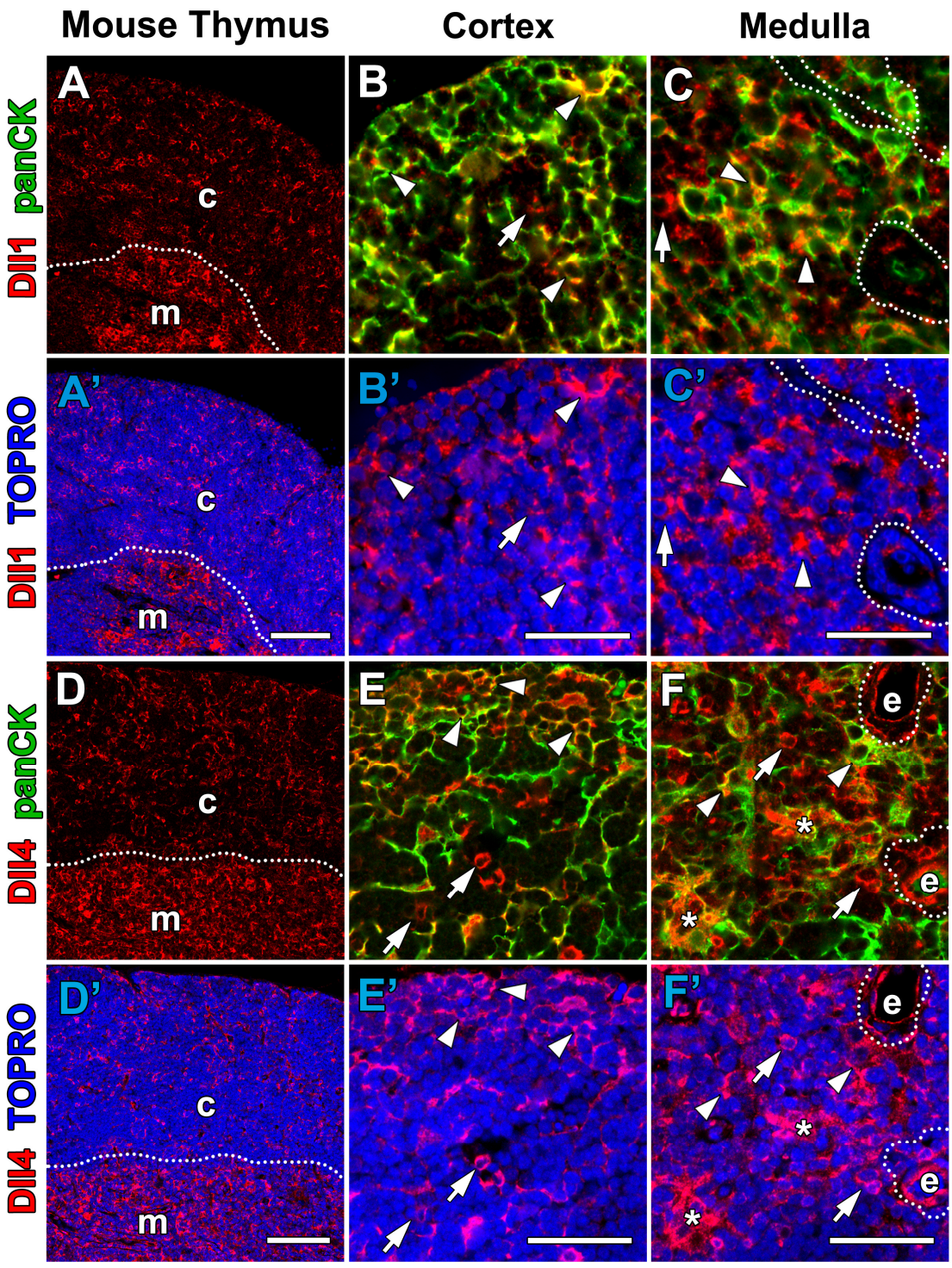


Figure 14. Dll1 and Dll4 Notch ligand expression in murine postnatal thymus. Images show FFPE sections of murine postnatal thymic tissue (< 16 week-old) stained for Dll1, Dll4 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *D,* General view of Dll1 and Dll4 distribution, respectively, in the murine thymus. Thymic regions are labeled as; *c:* cortex; and *m:* medulla; *dotted line*: CMJ. Scale bar: 100µm. *B*, *E*, Dll1 and Dll4 expression at the cortex, respectively. *C*, *F*, Dll1 and Dll4 expression at the medulla, respectively. Arrowheads indicate Dll1 or Dll4 expression in TECs (panCK+). Arrows indicate Dll1 or Dll4 expression in non-epithelial (panCK-) cells. *Dotted line:* PVS; *asterisks:* Thymic cysts; *e:* endothelium. Scale bars: 50µm. Images shown are representative of results obtained in $N \geq 3$ different tissue samples per ligand.

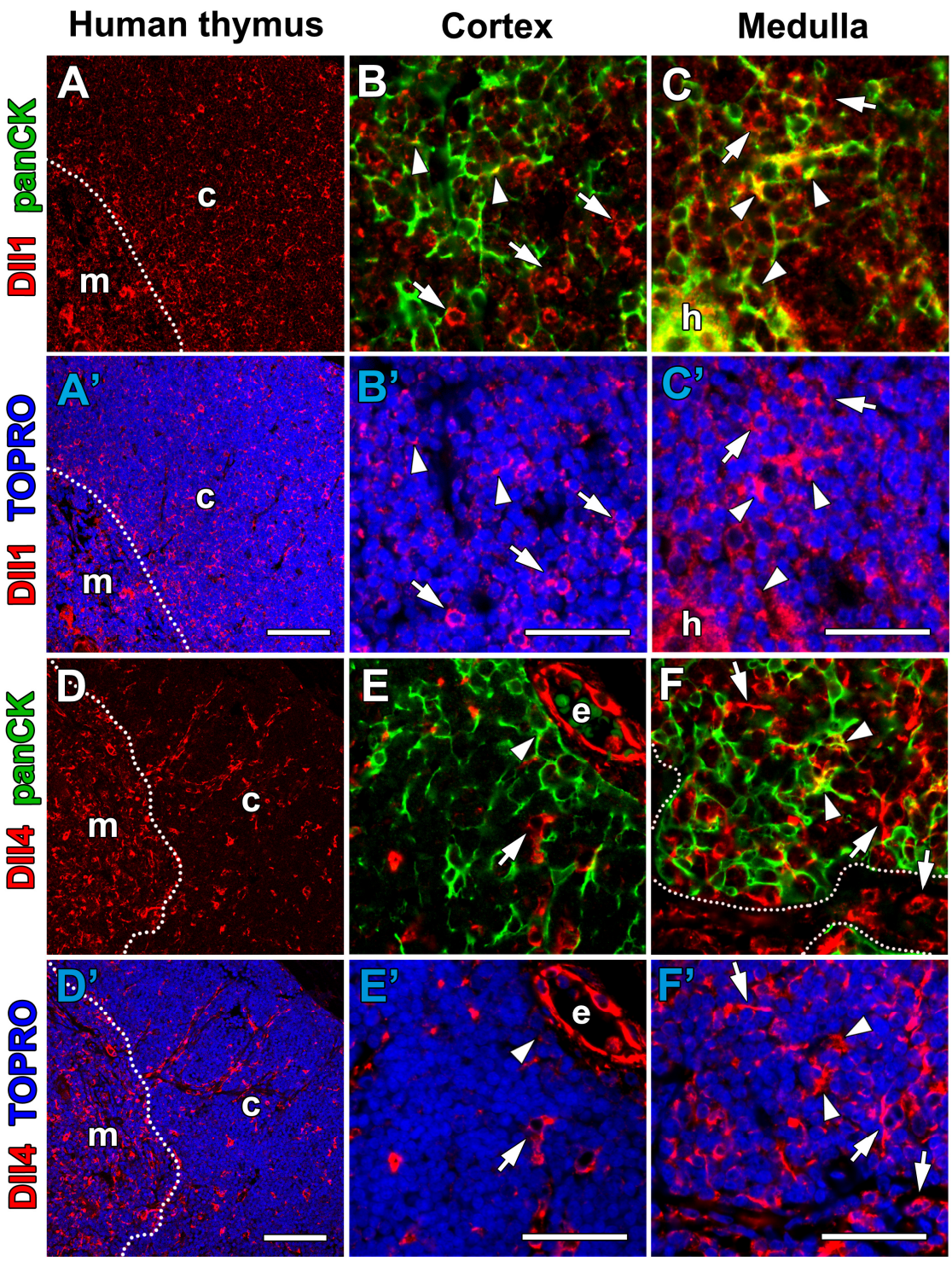


Figure 15. Dll1 and Dll4 Notch ligand expression in human postnatal thymus. Images show FFPE sections of human postnatal thymic tissue (< 17 months-old) stained for Dll1, Dll4 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *D,* General view of Dll1 and Dll4 distribution, respectively, in the human thymus. Thymic regions are labeled as *c:* cortex; and *m:* medulla; *dotted line:* CMJ. Scale bar: 100µm. *B*, *E*, Dll1 and Dll4 expression at the cortex, respectively. *C*, *F*, Dll1 and Dll4 expression at the medulla, respectively. Arrowheads indicate Dll1 or Dll4 expression in TECs (panCK+). Arrows indicate Dll1 or Dll4 expression in non-epithelial (panCK-) cells. *Dotted line:* PVS; *h:* Hassal's corpuscles; *e:* endothelium. Scale bars: 50µm. Images shown are representative of results obtained in $N \geq 3$ different tissue samples per ligand.

1.1.3. Notch ligands expression at the thymic medulla.

The thymic medulla is rich in Notch ligands. The four Notch ligands present in the thymus are found in this region, although they are differentially expressed by diverse cell types. In both human and mouse, Dll1 is highly expressed almost exclusively by mTECs, with the highest expression levels corresponding to mTECs near of or forming Hassal's corpuscles (Fig. 15C; 19B), also known as thymic cysts in mice (Fig. 14C). Additionally, some rare non-epithelial medullary cells do express Dll1. Owing to its absence from the cortex, Dll4 could be considered as a medullary ligand in the human thymus (Fig. 15D). Scarce mTECs expressed Dll4 at the medulla (Fig. 15F; 19B) but importantly, Dll4 is highly expressed at extraparenchymal sites, such as the PVS, and non-epithelial cells at the medulla (Fig. 15F and see 1.2.2 below), a different expression pattern to that found in the murine thymus medulla, where Dll4 is more notably expressed by mTECs (Fig. 14F).

Regarding Jagged ligands, Jag1 is a medullary-specific ligand in both mouse (Fig. 16A) and human thymus (Fig. 17A). Importantly, Jag1 is the only Notch ligand restricted to a specific anatomic region, and therefore it can be used as a formal medullary marker. In the medulla, Jag1 is abundantly expressed by mTECs, some non-epithelial cells, and by the vasculature (Fig. 17C; 19B). In contrast, Jag2 has a limited representation at the medulla, where it is almost absent in mouse (Fig. 16D, F) and is expressed by few mTECs and scarce non-epithelial cells in humans (Fig. 17D, F; 19B).

In summary, although Dll4 has been identified as the essential and non-redundant cortical Notch ligand required for T cell specification in mice (Koch et al., 2008) (Hozumi et al., 2008), our results show that Dll4 is absent from the human postnatal thymic cortex, where its expression is essentially restricted to blood vessels (Petrie, 2002). Instead, other ligands have a prominent expression by human cTECs, such as Jag2, followed by Dll1. Interestingly, Jag2 expression in cTECs is regulated in different cortical niches. Our studies also indicate that Jag1 displays the most restricted and conserved thymic expression pattern, as it is exclusively expressed at the medulla by mTECs in both mouse and humans. **Therefore, it is important to note that Jag1 and Jag2, two ligands of the same family, which are expected to have redundant functions, do define reciprocal microenvironments in the human thymus, i.e., thymic medulla and cortex, respectively (Fig. 18), which suggests putative functional differences.**

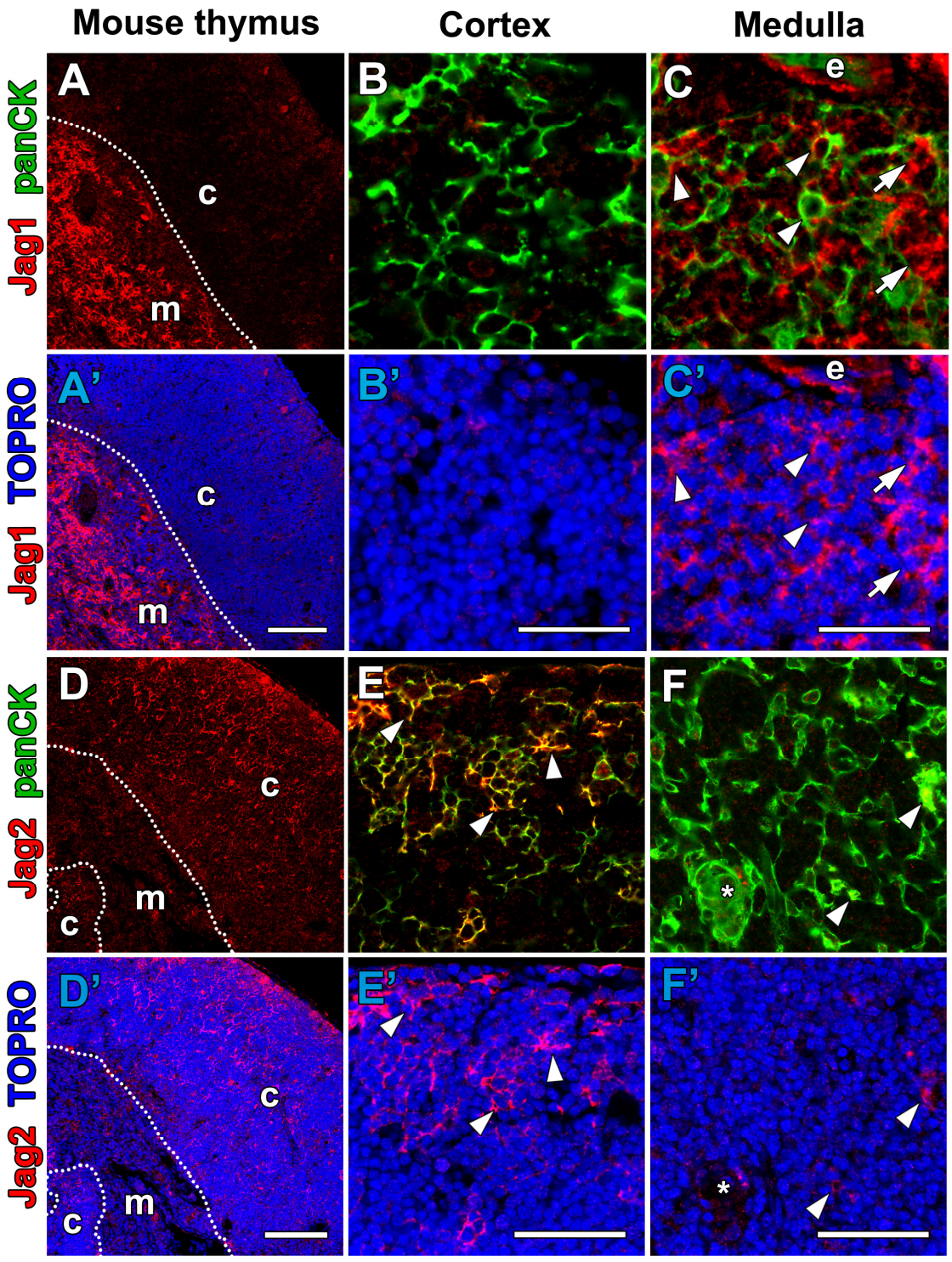


Figure 16. Jag1 and Jag2 Notch ligand expression in murine postnatal thymus. Images show FFPE sections of murine postnatal thymic tissue (< 16 weeks-old) stained for Jag1, Jag2 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *D,* General view of Jag1 and Jag2 distribution, respectively, in the murine thymus. Thymic regions are labeled as: *c:* cortex; and *m:* medulla; *dotted line:* CMJ. Scale bar: 100μm. *B*, *E*, Jag1 and Jag2 expression at the cortex, respectively. *C*, *F*, Jag1 and Jag2 expression at the medulla, respectively. Arrowheads indicate Jag1 or Jag2 expression in TECs (panCK+). Arrows indicate Jag1 or Jag2 expression in non-epithelial (panCK-) cells; *e:* endothelium; *asterisks:* Thymic cysts. Scale bars: 50μm. Images shown are representative of results obtained in $N \geq 3$ tissue samples per ligand.

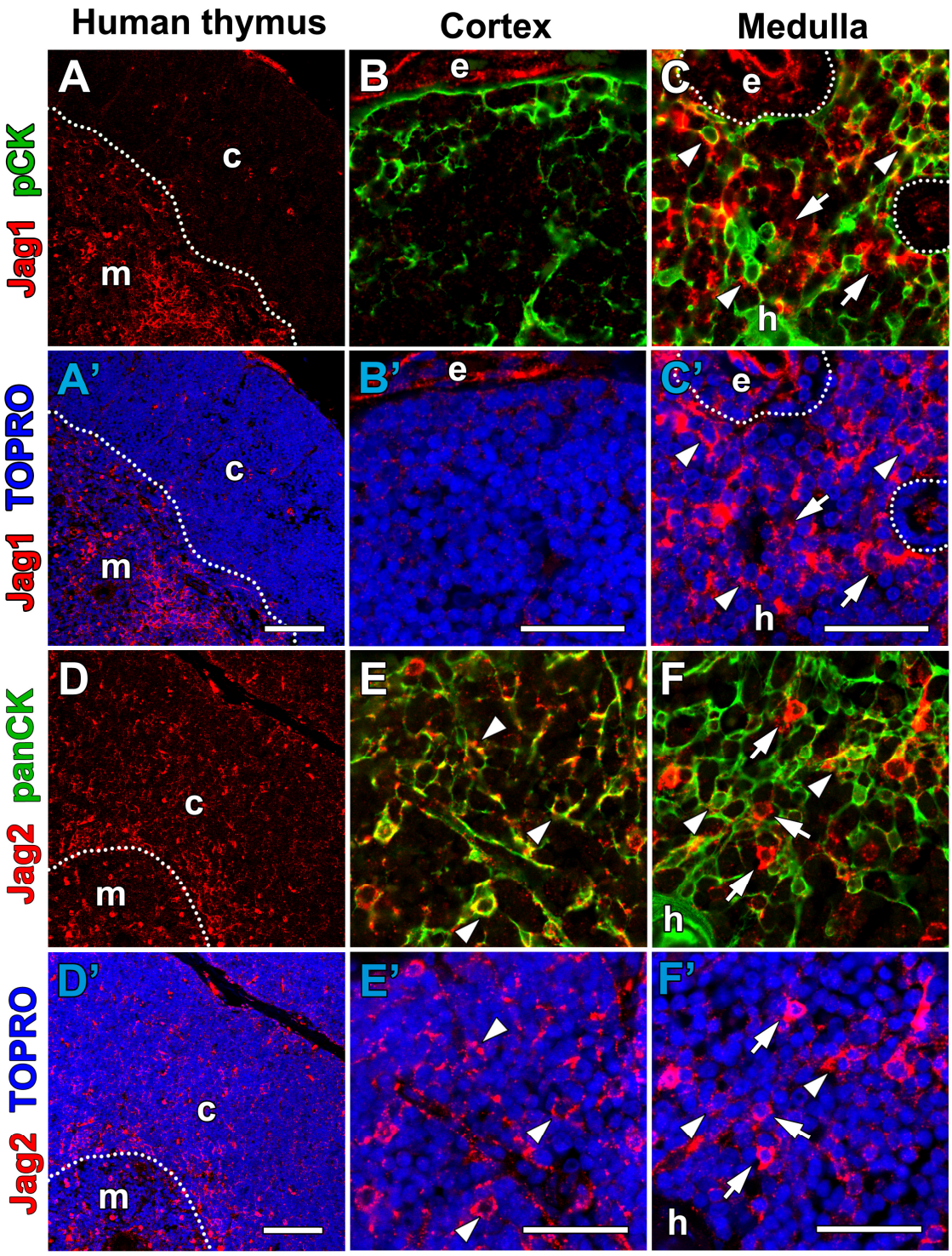


Figure 17. Jag1 and Jag2 Notch ligand expression in human postnatal thymus. Images show FFPE sections of human postnatal thymic tissue (< 17 month-old) stained for Jag1, Jag2 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *D*, General view of Jag1 and Jag2 distribution, respectively, in the human thymus. Thymic regions are labeled as: *c:* cortex; and *m:* medulla; *dotted line:* CMJ. Scale bar: 100μm. *B*, *E*, Jag1 and Jag2 expression at the cortex, respectively. *C*, *F*, Jag1 and Jag2 expression at the medulla, respectively. Arrowheads indicate examples of Jag1 or Jag2 expression in TECs (panCK+). Arrows indicate Jag1 or Jag2 expression in non-epithelial (panCK-) cells. *Dotted line:* PVS; *h:* Hassal's corpuscles; *e:* endothelium. Scale bars: 50μm. Images shown are representative of results obtained in $N \geq 3$ different tissue samples per ligand.

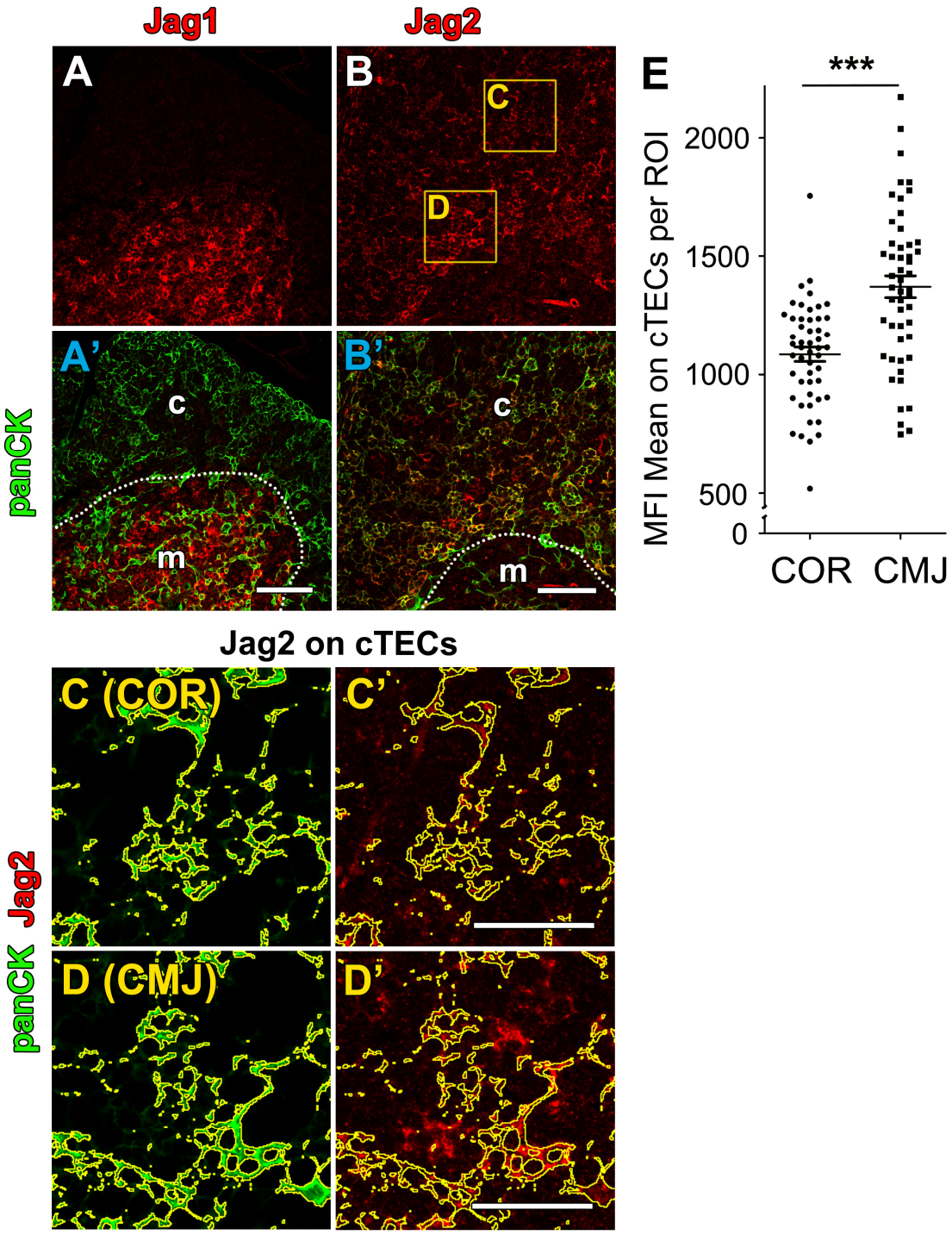


Figure 18. Jag1 and Jag2 Notch ligands define reciprocal microenvironments in the human thymus. Images show FFPE sections of human postnatal thymic tissue (< 17 months-old). *A*, *B*, Jag1 and Jag2 staining (red), respectively, plus panCK (green) staining showing co-localization (yellow) of Jag1 with mTECs and Jag2 with cTECs. Thymic regions are labeled as *c:* cortex; and *m:* medulla; *dotted line:* CMJ. Scale bars: 100µm. Images are representative of results obtained in $N \geq 3$ different tissue samples per ligand. *C*, *D,* ROIs (116µm x 116µm; $N = 45$) obtained from Jag2-stained tissue images of inner cortical and subcapsular (COR) or corticomedulary regions (CMJ) ($N \geq = 10$ per sample; $N = 3$ samples). PanCK staining allowed to create a selection area (yellow line) in cTECs by using Otsu's thresholding algorithm. cTEC-specific ROIs where used to obtain Jag2 Mean Fuorescence Intensity (MFI) values (C', D'). Scale bars: 50µm. *E*, Jag2 MFI values in cTECs at the COR and CMJ regions. All images were acquired in 12-bit with the same non-saturating settings. ***, $p < 0{,}001$.

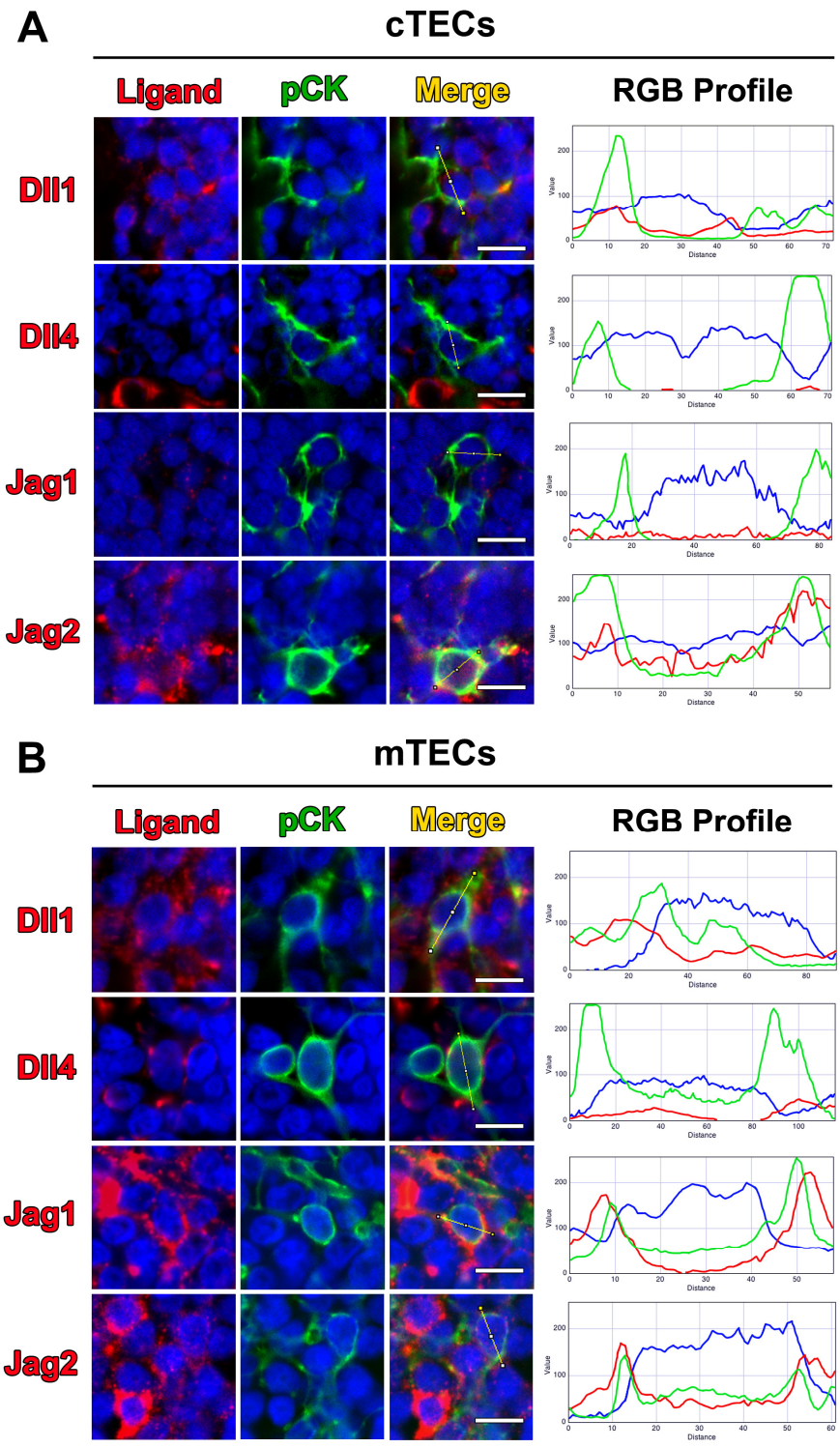


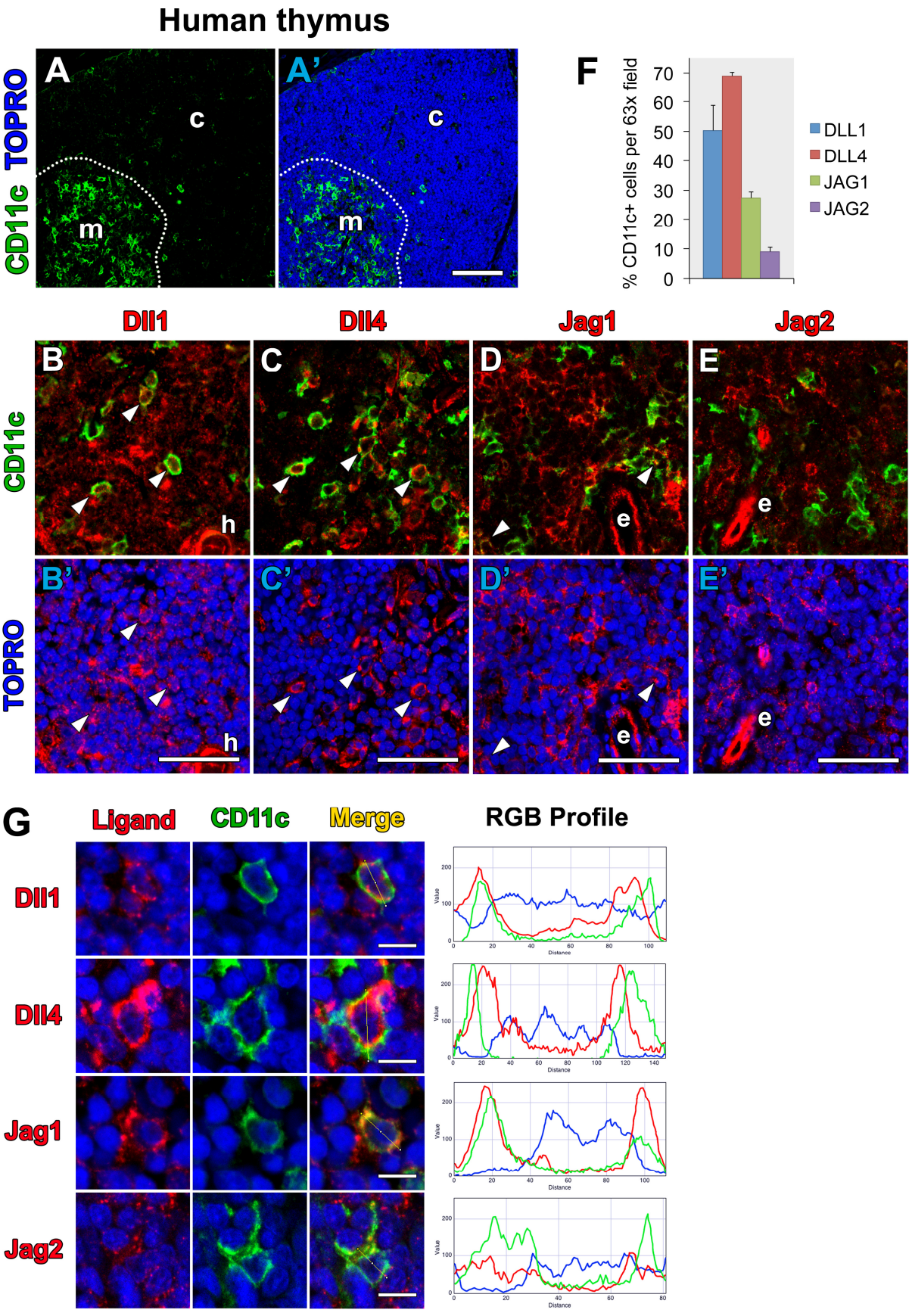
Figure 19. RGB co-localization profiles of Notch ligands win panCK in cTECs and mTECs. *A,* Image crops (30mm) of FFPE sections of the human thymus cortical region stained for Notch ligands. *B,* Image crops (30□m) of FFPE sections of human thymus medulla stained for Notch ligands. PanCK, Notch ligands and Topro3 MFI of isolated TECs was measured by tracing a linear ROI (yellow line) along the cells and RBG profiles (*right*) were plotted for each Notch ligand. Images are representative of results obtained in $N \geq 3$ different tissue samples per ligand. Scale bars: 10µm.

1.2. Non-epithelial thymic stromal cells also express Notch ligands in the human thymus.

We have identified an important number of non-epithelial cells in the human thymus, which are morphologically distinct from thymocytes, and express Notch ligands *in vivo*. As the most important thymic non-epithelial stromal cells include macrophages, dendritic cells and mesenchymal cells, we addressed the expression of Notch ligands on such stromal cells characterized by expression of either the myeloid marker CD11c, or the endothelial and mesenchymal marker CD34.

1.2.1. Notch ligands expression by myeloid CD11c+ cells.

Intrathymic macrophages can be found in both the cortex and medulla, where they are in charge of clearing death thymocytes resulting from selection processes (Surh and Sprent, 1994). Conversely, DCs are located exclusively at the medulla, where they play a capital role in the establishment of central tolerance (Savchenko et al., 2006). Therefore, the thymic medulla is enriched in myeloid cells that express the CD11c marker (Fig. 20A). In addition to CD11c+ conventional DCs (cDCs), the medulla contains plasmacytoid DCs (pDCs) positive for the CD123 marker. We attempted to characterize Notch ligands expression on these CD11c-CD123+ pDCs, but lack of a suitable anti-CD123 antibody frustrated our effort. We thus aimed at characterizing Notch ligand expression on intrathymic CD11c+ cells and found that they do express all four Notch ligands (Fig. 20G) although with different frequencies (Fig. 20F). We calculated total CD11c+ cells and CD11c+ cells expressing Notch ligands per field in $N \geq 10$ images (63x) from $N = 3$ different thymic tissue samples, and observed that Delta family ligands are expressed in a major CD11c+ cell subset (>50% Dll1; >70% Dll4; Fig. 20B, C, F), while Jag1 and Jag2 ligands are poorly expressed by CD11c+ cells (less than 20% and 10% respectively; Fig. 20D, E, F). Further RGB profiling on isolated CD11c+ cells confirmed these findings (Fig. 20G).


Human thymus
CD11c TOPRO
A
A'
c
m
F
% CD11c+ cells per 63x field
70
60
50
40
30
20
10
0
DLL1
DLL4
JAG1
JAG2
Dll1
Dll4
Jag1
Jag2
CD11c
TOPRO
B
C
D
E
h
e
B'
C'
D'
E'
G
Ligand
CD11c
Merge
RGB Profile
Dll1
Dll4
Jag1
Jag2
Value
Distance

<< Figure 20. Intrathymic CD11c+ myeloid stromal cells express Delta family Notch ligands. Images show FFPE sections of human postnatal thymic tissue (< 17 months-old) stained with the myeloid marker CD11c (green) and Notch ligands (red). Topro3 was used for nuclear staining (blue). *A,* General view of CD11c expression in the human thymus. Thymic regions are labeled as *c:* cortex; and *m:* medulla; *dotted line:* CMJ. Scale bar: 100µm. *B, C, D*, *E* show CD11c and Dll1, Dll4, Jag1 and Jag2 Notch ligand staining, respectively, at the thymic medulla. Arrowheads indicate Notch ligands expression on CD11c+ cells. CD11c co-localization (yellow) is remarkable with Delta family ligands, while scarce with Jagged ligands. *h:* Hassal's corspuscles; *e:* endothelium. Scale bars: 50µm. *F,* Bar graph represents the mean percentage of CD11c+ cells encountered per 63x field expressing each Notch ligand. *G, left,* Image crops (30µm) of FFPE sections of the human thymus medulla stained for CD11c, Notch ligands and Topro3. *Right,* RGB co-localization profiles of CD11c with Notch ligands and Topro3. MFI values of isolated CD11c+ cells were measured by tracing a linear ROI (yellow line) along the cells and. Scale bars: 10µm. Data are representative of results obtained in $N \geq 3$ different tissue samples per ligand.

1.2.2. Notch ligands expression by endothelial and mesenchymal cells at the perivascular space.

The CMJ region is often disrupted by perivascular regions from where HPCs can enter the thymus (Mori et al., 2007) (Fig. 4A). The intrathymic perivascular space (PVS) has been characterized as the third anatomical region of the thymus in addition to the cortex and medulla. It is a region separated by two basement membranes: one membrane is associated with a blood vessel, while the other is associated with the epithelial border (Kato and Schoefl, 1989). We have identified this region by the absence of epithelial markers (panCK) and by positive staining for CD34 (Abe et al., 2011) (Kim et al., 2014) (Fig. 21). The PVS structure includes mesenchymal tissue, endothelial cells, and myeloid cells. Endothelial cells of vascular walls and mesenchymal cells forming the PVS may provide early signaling cues to HPCs migrating into and/or seeding the thymus (Mori et al., 2007), which may play an important role in T cell development. It is known that Notch is a major pathway involved in endothelial cell function (Gridley, 2010), and the vasculature is rich in Notch signaling components. Indeed, we have observed that thymic vasculature expresses Dll4, Jag1 and Jag2 (Fig. 22B, C, D), but while Dll4 is expressed by the entire thymic vasculature including arteries, venules and capillaries, Jag1 and Jag2 are present during development and some endothelial cells presumably of arterial fate in the adult (Villa et al., 2001). Additionally, CD34+ mesenchymal cells surrounding blood vessels express Dll4 and some Jag1 (Fig. 22B, C). Conversely, Dll1 and Jag2 are not expressed by CD34+ perivascular cells (Fig. 22A, D). However, Dll1 expression can be detected in some CD34- cells (Fig. 22A), which may represent SP thymocytes leaving the thymus (Mori et al., 2007). Therefore, **vascular endothelium as well as different PVS cell types at the CMJ region provide an important source of Dll4 to progenitors entering the human postnatal thymus, suggesting that they may contribute to Notch activation at very early T cell developmental stages.**

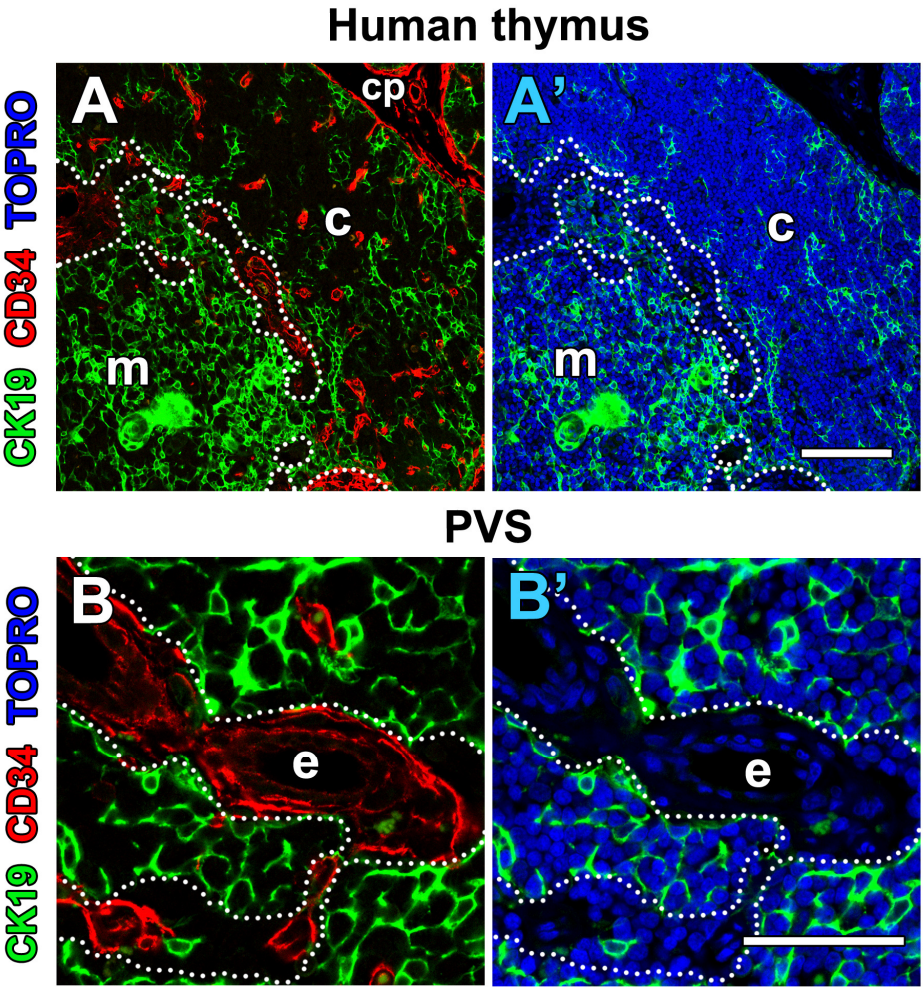


Figure 21. Immunohistological definition of the human perivascular space. FFPE tissue sections of human postnatal thymus (< 17 months-old) were stained with the epithelial marker cytokeratin 19 (CK19) (green) and the endothelio-mesenchymal and hematopoietic progenitor cell marker CD34 (red). Topro3 was used for nuclear staining (blue). *A*, General view of CD34 expression in the human thymus. The perivascular space (PVS), defined as CK19- CD34+ tissue, is indicated by a dotted line. Thymic regions are labeled as *c:* cortex; and *m:* medulla; *cp:* thymic capsule. Scale bar: 100µm. *B,* PVS detail. *e: endothelium.* Images are representative of results obtained in $N \geq 3$ different tissue samples. Scale bar: 50µm.

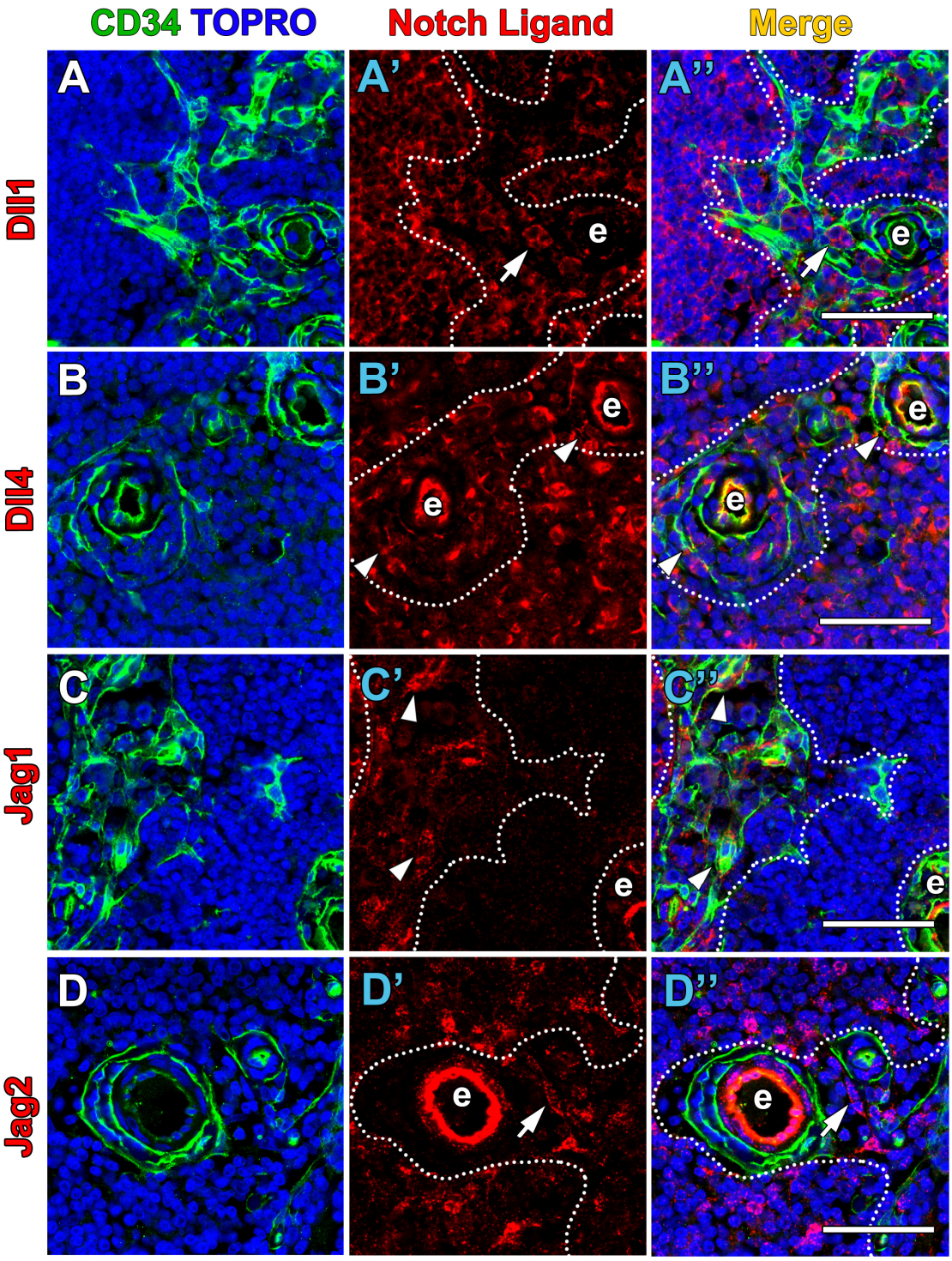


Figure 22. Notch ligands are expressed by thymic perivascular cells and thymic endothelium. FFPE sections of human postnatal thymic tissue (< 17 months-old) twere stained for Dll1, Dll4, Jag1 and Jag2 (red) and CD34 (green). Topro3 was used for nuclei counterstaining (blue). *A*, *B*, Dll1 and Dll4 expression, respectively, on human thymic endothelium and perivascular cells. Dll1 is only detected in CD34- perivascular cells (arrows), while Dll4 is expressed in mesenchymal CD34+ (arrowheads) cells and endothelium *(e).* C, D, Jag1 and Jag2 expression, respectively, on human thymic endothelium and perivascular cells. Jag1 and Jag2 are expressed by endothelium (e), but only Jag1 is expressed by perivascular CD34+ cells (arrowheads). PVS limits are indicated by a dotted line. Images are representative of results obtained in $N \geq 2$ different tissue samples per ligand. Scale bars in all images represent 50µm.

Finally, to provide an accurate picture of Notch ligand expression by human thymic stroma, we have performed quantitative studies of Notch ligand expression on cortical and medullary TECs (panCK+) and myeloid cells (CD11c+) by using the co-localization coefficient of Mander's. The ICA (Intensity Colocalization Analysis) plugin (Li et al., 2004) (see Materials and Methods) was applied to a $N \geq 10$ cortical and $N \geq 10$ medullary images (63x) for each stromal marker and Notch ligand, obtained from $N = 3$ different postnatal tissue samples. M1 and M2 Mander's coefficients were obtained. M1 coefficients represented in Fig. 23 formally confirmed the findings shown by the co-expression analyses described above. **Altogether, these results demonstrate that Notch ligands are differentially distributed *in vivo* in different histological and functional regions of the human postnatal thymus.**

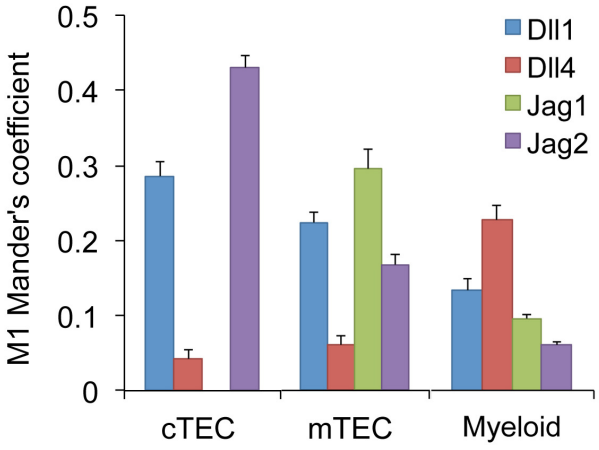


Figure 23. Mander's coefficient of Notch ligands expression in human thymic stromal cells. Bar graph diagram represents the M1 co-localization coefficient of Mander obtained by applying the Intensity Colocalization Analysis (ICA) plugin to $N \geq 10$ images from human thymic cortex and medulla stained with Notch ligands and either panCK or CD11c. Results obtained in $N \geq 3$ different tissue samples per ligand are shown.

1.3. Temporal regulation of Notch ligand expression during human thymus ontogeny.

1.3.1. Dll4 is early expressed by cTECs during human thymus ontogeny.

The finding that cTECs of the postnatal human thymus, in contrast to their murine counterparts, lack Dll4 expression prompted us to investigate the possibility that intrathymic niches, and particularly Notch ligand expression, may vary along time, as has been shown to occur during thymus involution (Haynes et al., 2000) (Aw et al., 2009). In fact, expression of Dll4 by cTECs is temporally regulated in mice, as Dll4 expression is high in the fetal and neonatal thymus, but decreases dramatically in the adult thymus (Fiorini et al., 2008). In order to assess temporal regulation of Notch ligand expression during human thymus ontogeny, we analyzed samples of human fetal thymuses ranging from 11 to 19 weeks of gestation. We found no substantial differences in Dll1 (Fig. 24A) or Jag1 (Fig. 25A) expression patterns between fetal and postnatal tissue samples. Dll1 was present in epithelial and hematopoietic cells from

the cortex and medulla, although its expression in fetal cTECs was more significant than in postnatal cTECs (Fig. 24B), and less significant in fetal mTECs than in postnatal mTECs (Fig. 24C), supporting previous observations of Dll1 expression up-regulation with age (Aw et al., 2009). Moreover, cortical Jag2 expression was restricted to cTECs, as in the postnatal thymus (Fig. 25E). Conversely, fetal medulla showed a remarkable higher number of Jag2+ non-epithelial cells than in the postnatal thymus (Fig. 25F). The nature of this Jag2+ fetal medullary cells is unknown, but it may correspond to either mature T cells or pDCs, as in the postnatal thymus no Jag2-expressing CD11c+ cells were found (Fig. 20E, F). Finally, analysis of Dll4 expression in the medulla of fetal thymus samples revealed a Dll4 expression pattern similar to that observed in the postnatal medulla (Fig. 24F). But interestingly, the fetal cortical expression pattern of Dll4 was different to that observed in the postnatal thymus as in the former Dll4 was notably expressed in cTECs (Fig. 24D, E). To quantitatively confirm a highest Dll4 expression in fetal cTECs versus postnatal cTECs in the human thymus, Dll4 MFI was measured specifically on cTECs by creating ROIs ($N \geq 30$) on panCK+ cells in $N \geq 10$ images (63x) per thymic sample ($N = 3$) (Fig. 26A). Direct comparison of total fetal and postnatal cTEC Dll4 MFI values, showed that Dll4 expression is significantly higher in fetal than in postnatal cTECs (Fig. 26C). But, in order to make accurate comparisons between tissue samples from different experiments, we used thymus endothelium, a well-known and stable Dll4-expressing tissue, as an internal control (Fig. 26B). Indeed, comparison of Dll4 MFI values expressed in fetal and postnatal thymic endothelium confirmed that Dll4 expression by thymic endothelial cells remains essentially constant during thymus ontogeny (Fig. 26C; $p = 0{,}458$). Therefore, epithelial Dll4 MFI values were normalized to endothelial Dll4 MFI values obtained from the very same sample (Fig. 26D). Accordingly, Epithelial/Endothelial Dll4 MFI Ratios (TEC/EC) showed more than 2-fold decreased Dll4 expression levels in postnatal cTECs when compared with fetal cTECs (Fig. 26D), thus confirming a highly significant downregulation of Dll4 in cTECs during human thymic ontogeny. Equivalent comparison of postnatal human and postnatal murine TEC/EC Ratios allowed us to confirm that Dll4 remains expressed by cTECs in the mouse postnatal thymus (Fig. 26D). **In conclusion, Dll4 expression in fetal but not in postnatal human cTECs suggests that Dll4 undergoes an early downregulation during human thymus ontogeny. As Dll4 downregulation has been also reported in mice at later postnatal ages (Fiorini et al., 2008), it seems that kinetics of Dll4 downregulation in cTECs varies between mouse and human.**

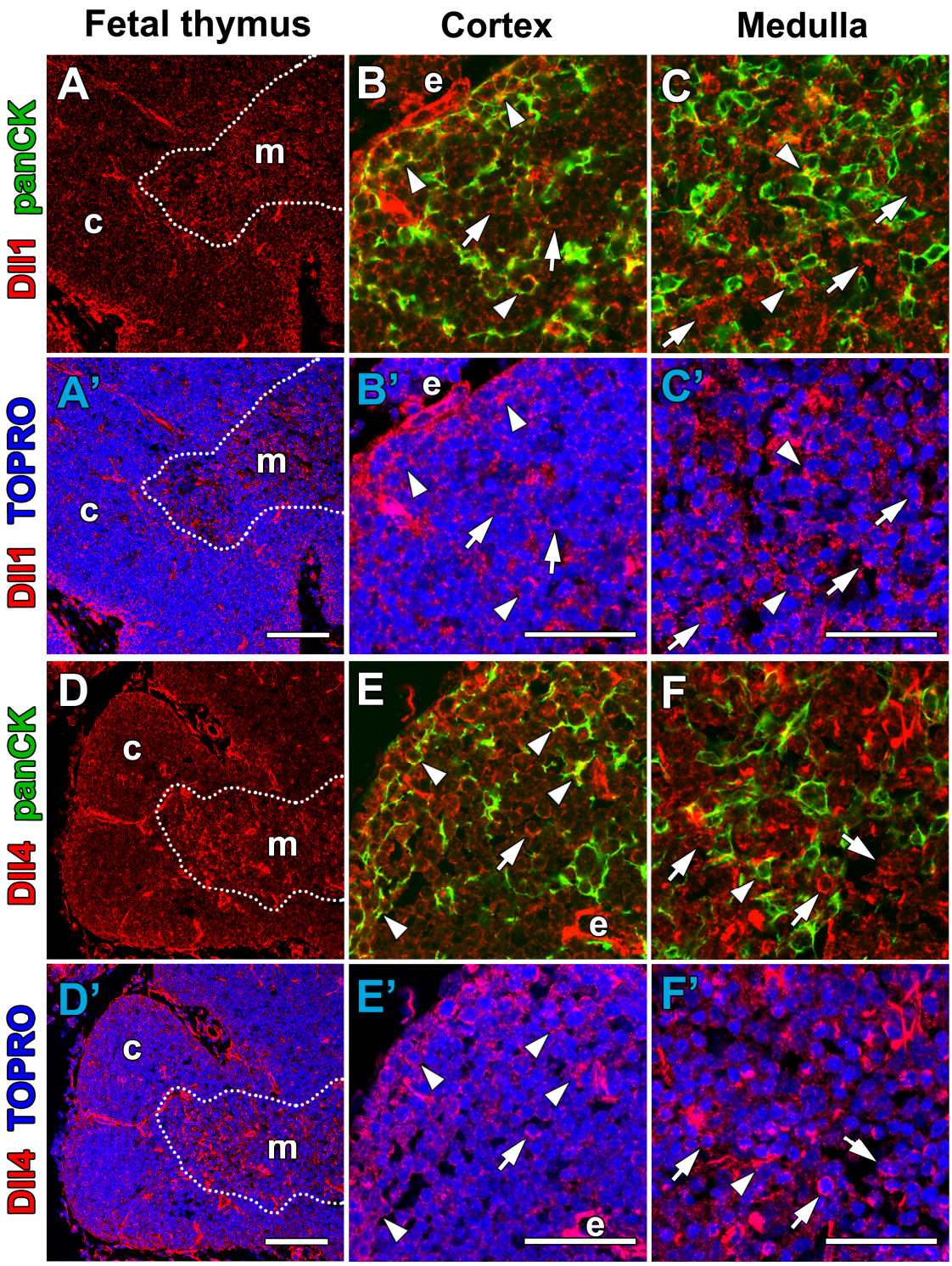


Figure 24. Dll1 and Dll4 Notch ligand expression in human fetal thymus. Images show FFPE sections of human fetal thymic tissue (11 - 19 weeks) stained for Dll1, Dll4 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *D,* General view of Dll1 and Dll4 distribution, respectively, in the human thymus. Thymic regions are labeled as *c:* cortex; and *m:* medulla; *dotted line:* CMJ. Scale bar: 100µm. *B*, *E*, Dll1 and Dll4 expression at the cortex, respectively. *C*, *F*, Dll1 and Dll4 at the medulla, respectively. Arrowheads indicate Dll1 or Dll4 expression by TECs (panCK+). Arrows indicate Dll1 or Dll4 expression by non-epithelial (panCK-) cells. *e:* endothelium. Scale bars: 50µm. Images shown are representative of results obtained in *N* = 2 different tissue samples per ligand.

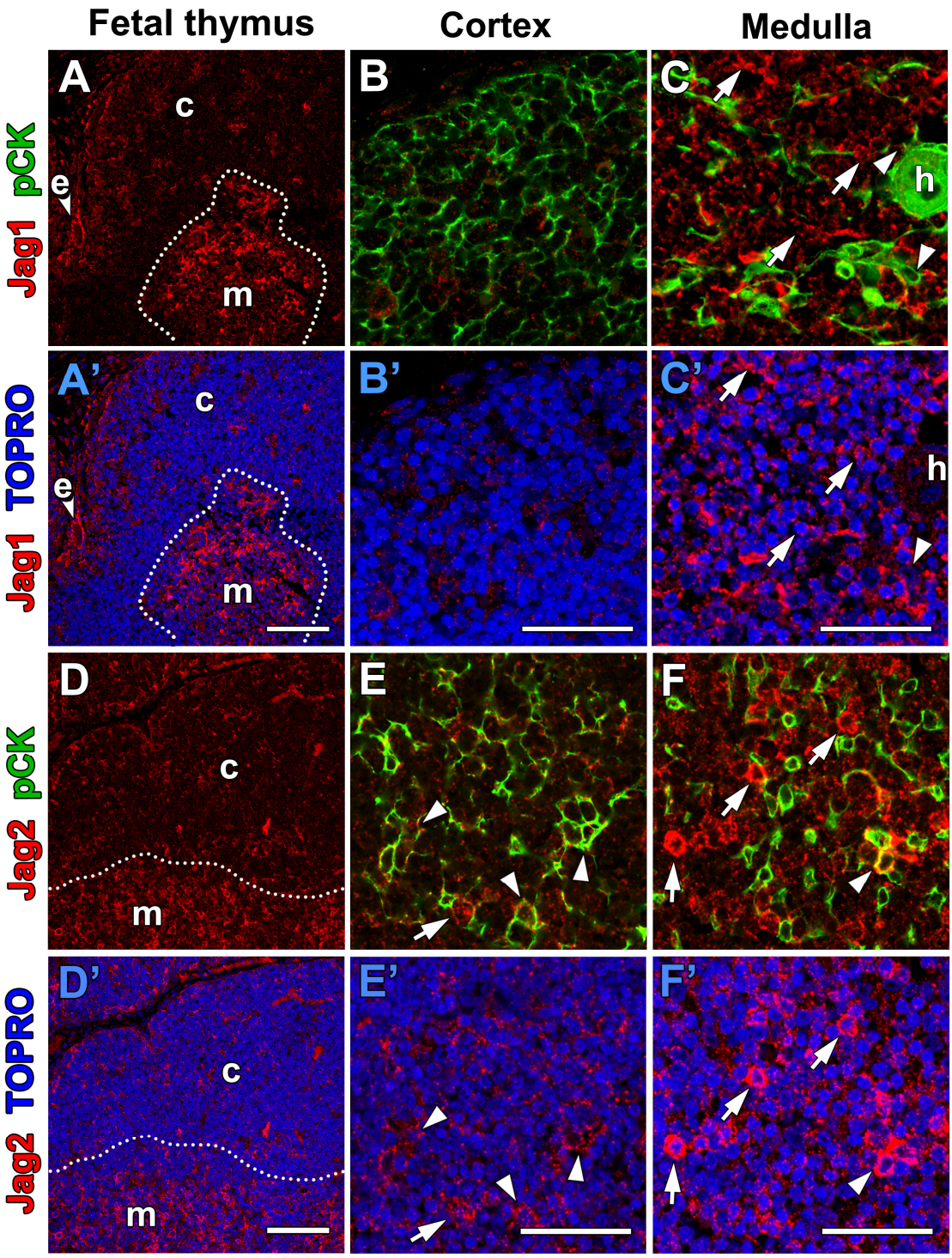


Figure 25. Jag1 and Jag2 Notch ligand expression in human fetal thymus. Images show FFPE sections of human fetal thymic tissue (11 - 19 weeks) stained for Jag1, Jag2 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *D,* General view of Jag1 and Jag2 distribution, respectively, in the human thymus. Thymic regions are labeled as *c:* cortex; and *m:* medulla; *dotted line:* CMJ. Scale bar: 100µm. *B*, *E*, Jag1 and Jag24 expression at the cortex, respectively. *C*, *F*, Dll1 and Dll4 at the medulla, respectively. Arrowheads indicate Jag1 or Jag2 expression by TECs (panCK+). Arrows indicate Jag1 or Jag2 expression by non-epithelial (panCK-) cells. *e:* endothelium. Scale bars: 50µm. Images shown are representative of results obtained in *N* = 2 different tissue samples per ligand.

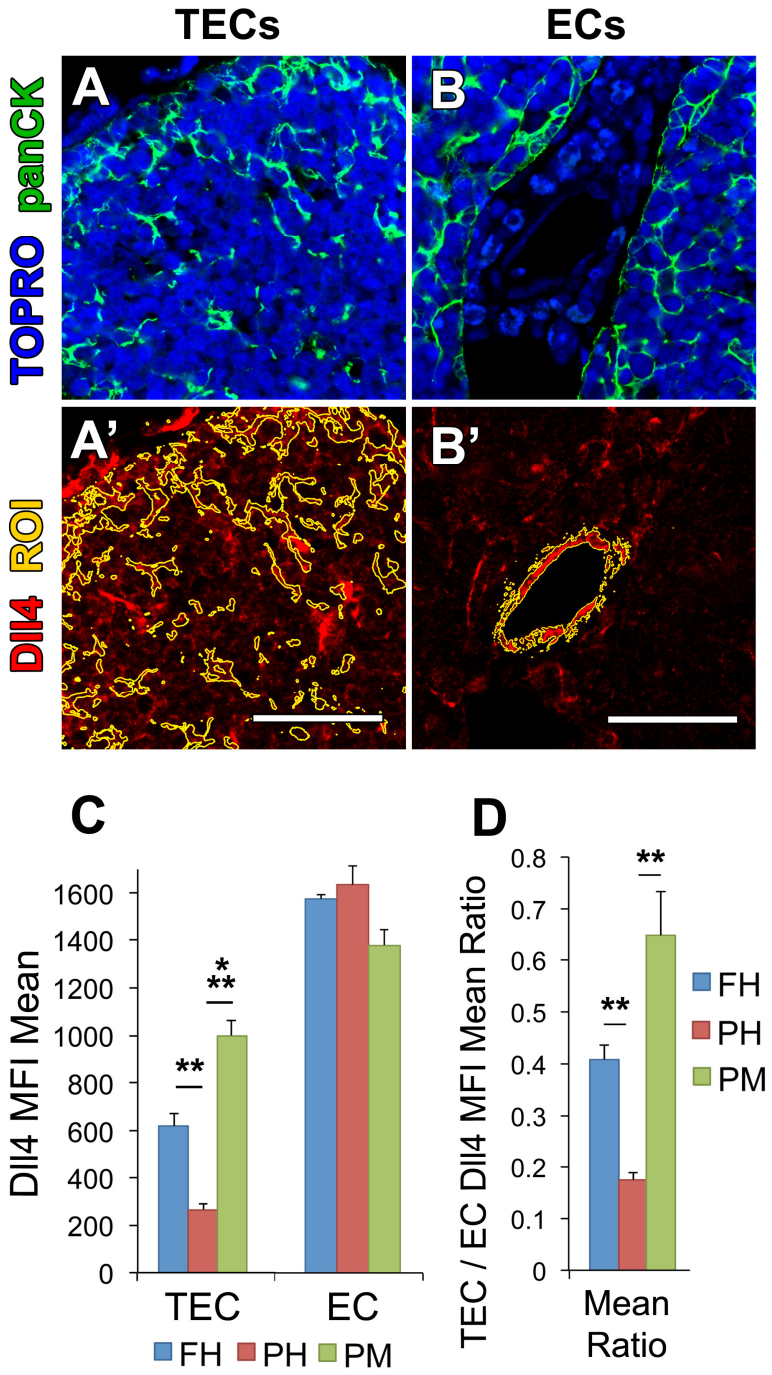
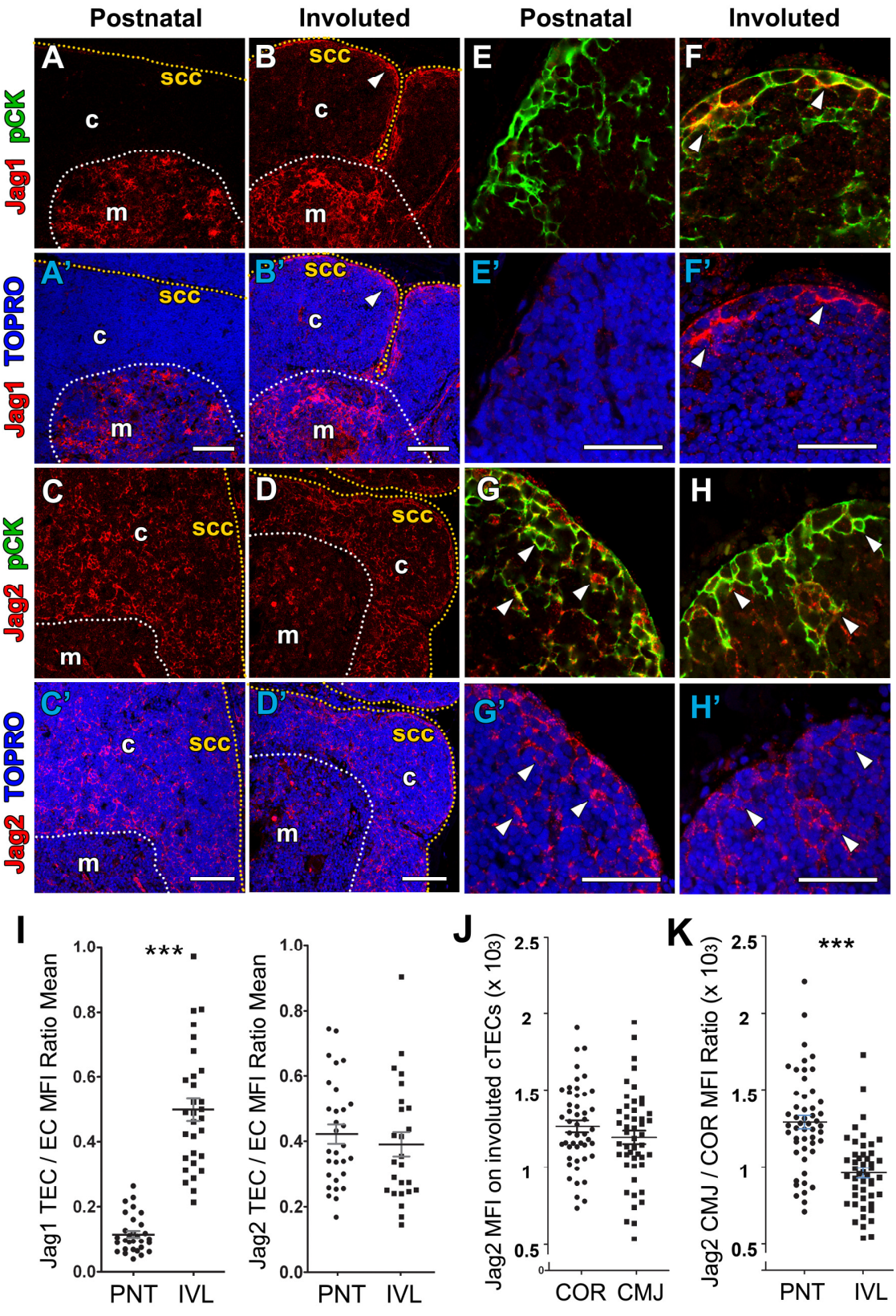


Figure 26. Quantification of Dll4 expression in fetal and postnatal cTECs. Images show FFPE sections of fetal human (11 - 19 weeks), postnatal human (< 17 month-old) and postnatal murine (< 16 weeks-old) thymic tissue stained for Dll4 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *B,* Dll4 quantification strategy based on ROIs (yellow lines) defined on TECs and endothelial cells (ECs). Scale bars: 50µm. *C*, Bar graphs showing Dll4 MFI in different cells. EC showns similar expression levels of Dll4 in mice and human tissue, and along age. EC Dll4 MFI values were obtained from $N \geq 10$ images per sample ($N = 3$ samples) and used to normalize cTEC Dll4 MFI values. All images were acquired in 12-bit color depth with the same non-saturating settings. *D*, Bar graph showing TEC/EC Dll4 MFI ratios in fetal human (FH), postnatal human (PH) and postnatal murine (Jiang et al.) thymus. Images shown are representative of results obtained in $N \geq 3$ different tissue samples. **, $p < 0,01$; ***, $p < 0,001$.

1.3.2. Jag1 expression at the SCC is upregulated with thymic involution.

We next wanted to address whether ageing and involution of the human thymus might involve changes in Notch ligand distribution. To this end, we analyzed Notch ligand expression in samples of human adult thymuses ranging from 11 to 15 years-old. Analysis of Delta ligands did not show any variation in their expression patterns when compared with neonatal thymus samples (data not shown), except an increased Dll1 expression in Hassall's corpuscles already observed when compared fetal versus postnatal thymi. However, we observed that Jagged ligands up-regulate their expression in cTECs located at the SCC region with age. As depicted in Fig. 27A, E, Jag1 is completely absent from the neonatal cortex, while it is nicely expressed by subcapsular cTECs of the involuted thymus (Fig. 27B, F). Likewise, a slight up-regulation is observed for Jag2 at the SCC (Fig. 27D, H). As thymic vasculature expresses Jag1 (Lehar et al., 2005) and Jag2 (personal observation), we quantified subcapsular epithelial and endothelial MFI in neonatal and involuted thymi in $N \geq 10$ images (63x) per thymic sample ($N = 3$), using the same method described for Dll4. Jag1 TEC/EC MFI Ratios were compared between neonatal and involuted thymi (Fig. 27I, left graph), revealing a highly significant increase in Jag1 expression in subcapsular cTECs with thymic involution. Regarding Jag2, as it was already expressed by subcapsular cTECs in the postnatal thymus (Fig. 27G), there was not significant upregulation with age (Fig. 27H, I, right graph; p = 0,6217).

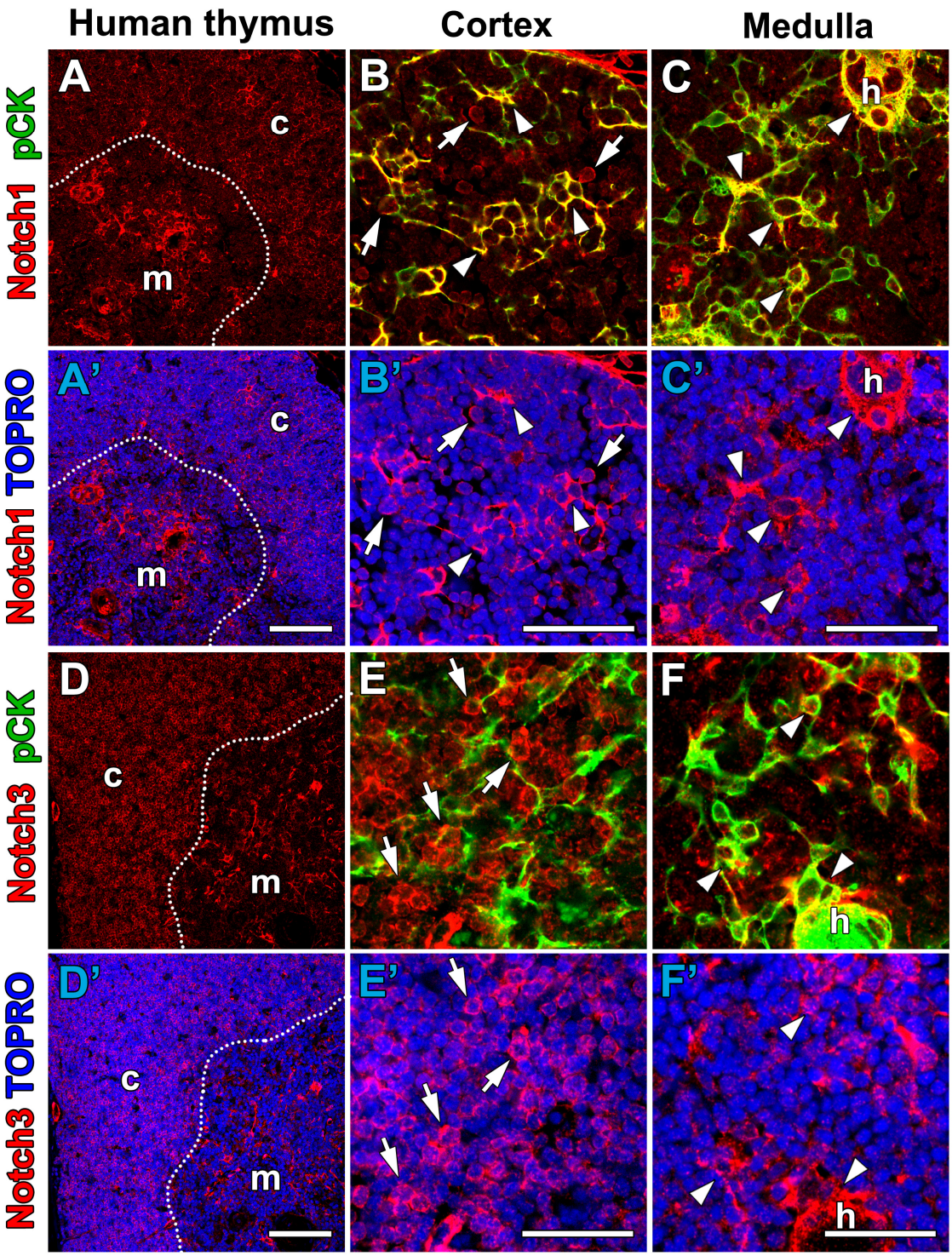
Although we found no significant upregulation of Jag2 at the SCC with age, we observed that the Jag2 enrichment found at the CMJ of the human postnatal thymus does not occur in the involuted thymus (Fig. 27D). Jag2 MFI values from involuted cTECs at the cortex (COR) or CMJ were obtained in the very same way as described in 1.1.2., and were statistically compared. MFI values obtained from both anatomic regions were not significantly distinct (Fig. 27J; p = 0,2254), indicating that Jag2 expression levels at the cortex and CMJ are similar in the involuted human thymus. In order to make an accurate comparison between tissue samples from different experiments, we next obtained Jag2 MFI CJM/COR Ratios ($N = 15$ images; 63x) from postnatal ($N = 3$) and involuted thymuses ($N = 2$). As depicted in Fig. 27K, Jag2 CMJ/COR MFI ratio is significantly lower in the involuted thymus, thus confirming that Jag2 enrichment at the CMJ niche is lost with thymic involution. **In summary, the early downregulation of Dll4 at the cortex, the upregulation of Jag1 at the SCC, and the lack of Jag2 enrichment at the CMJ observed in involuted thymus samples confirm that Notch ligand expression is regulated in cTECs in a temporal-manner.**


Postnatal
Involuted
Postnatal
Involuted
Jag1 pCK
Jag1 TOPRO
Jag2 pCK
Jag2 TOPRO
A
B
E
F
A'
B'
E'
F'
C
D
G
H
C'
D'
G'
H'
scc
c
m
I
Jag1 TEC / EC MFI Ratio Mean
Jag2 TEC / EC MFI Ratio Mean
***
PNT
IVL
J
Jag2 MFI on involuted cTECs (x 10³)
COR
CMJ
K
Jag2 CMJ / COR MFI Ratio (x 10³)

<< Figure 27. Jag1 and Jag2 expression in involuted human thymus. Images show FFPE sections of human postnatal (< 17 months-old) and human involuted (> 8 years-old) thymus samples were stained for Jag1 and Jag2 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *B, C, D,* General view of Jag1 and Jag2 expression in postnatal and involuted human thymus. Thymic regions are labeled as *scc:* subcapsular cortex; *c:* cortex; and *m:* medulla. *Dotted line:* CMJ; *Yellow dotted line*: thymic capsule. Scale bars: 100µm. *E*, *F*, Detailed images of Jag1 expression at the SCC of postnatal and involuted thymus, respectively. *G*, *H*, Detailed images of Jag2 expression at the SCC of postnatal and involuted thymus, respectively. Arrowheads indicate subcapsular cTECs expressing Jag1 or Jag2 ligands. Scale bars: 50µm. Images shown are representative of results obtained in $N \geq 2$ different tissue samples per ligand. *I,* Quantification of Jag1 and Jag2 expression on subcapsular cTECs of postnatal (PNT) and involuted (IVL) human thymus. Similarly to that described for Dll4. EC Jag1 and Jag2 MFI values were obtained by image thresholding from $N \geq 15$ images per sample, and used to normalize MFI Jag1 and Jag2 values of subcapsular cTECs from postnatal ($N = 3$) and involuted thymus ($N = 2$). TEC/EC MFI ratios means are shown. All images were acquired in 12-bit color depth with the same non-saturating settings. *J*, COR and CMJ Jag2 MFI mean values in postnatal and involuted thymus samples. Values were defined based on cortical (COR) or corticomedulary (CMJ) ROIs (116µm x 116µm; $N = 45$) obtained from images (25x; $N \geq 10$ per thymic sample) of Jag2-stained thymus samples (N = 2). *K*, CMJ/COR Jag2 MFI Jag2 values of postnatal and involuted thymus. Results shown are representative of $N \geq 15$ images per sample ($N = 3$ postnatal; $N = 2$ involuted samples).

1.4. Notch1, Notch3 and Notch4 receptors are differentially expressed in the human thymus.

We have demonstrated that the main stromal components of the thymus express Notch ligands in discrete microenvironments subjected to spatial and temporal regulation. The other side of the coin corresponds to Notch receptors. Nothing is known about Notch receptor expression by stromal cells, but it has been demonstrated that thymocytes actively regulate Notch receptor expression along development (Hasserjian et al., 1996) (Fiorini et al., 2009). As detailed in the introduction, Notch1 is the essential and non-redundant receptor needed for T-cell specification. Notch1 expression has been described to be concentrated within the outer cortical area of the postnatal murine thymus (Fiorini et al., 2009), but little is known about the intrathymic distribution of other Notch receptors. We know that Notch1, Notch2 and Notch3 transcripts are detected in thymocytes and TECs in mice. Besides, thymocytes interacting with TECs expressing Notch ligands up-regulate Notch3 after T cell commitment, suggesting that Notch3 may be involved in thymocyte development after Notch1-induced T cell specification (Felli et al., 1999) (Shi et al., 2011).


Human thymus
Cortex
Medulla
Notch1 pCK
Notch1 TOPRO
Notch3 pCK
Notch3 TOPRO
A
B
C
A'
B'
C'
D
E
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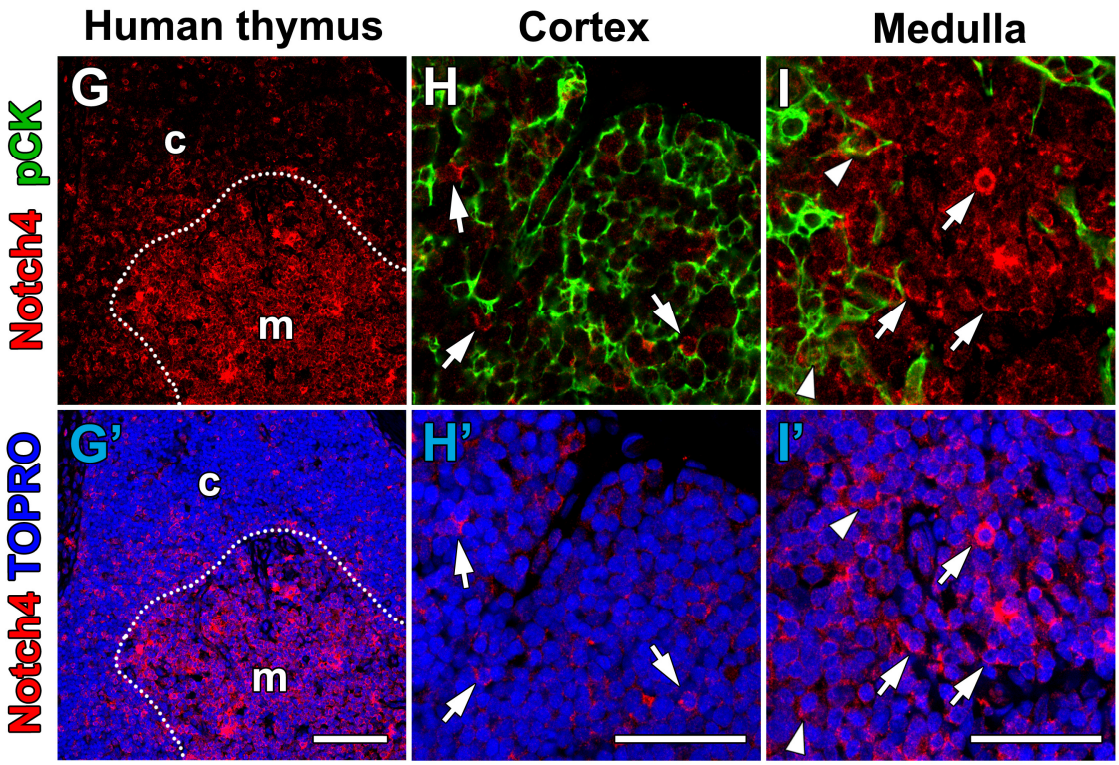
E'
F'
c
m
h



<<^^Figure 28. Notch1, Notch3 and Notch4 expression in human postnatal thymus. Images show FFPE sections of human postnatal thymic tissue (< 17 months-old) stained for Notch1, Notch3, Notch4 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *D, G,* General view of Notch1, Notch3 and Notch4 distribution in the human thymus. Thymic regions are labeled as *c:* cortex; and *m:* medulla. *Dotted line:* CMJ. Scale bars: 100µm. *B*, *E*, *H,* Notch1, Notch3 and Notch4 expression, respectively, at the cortex. *C*, *F*, Notch1, Notch3 and Notch4 expression, respectively, at the medulla. Arrowheads indicate Notch receptors expression by TECs (panCK+) and arrows indicate expression by non-epithelial (panCK-) cells. *h:* Hassal's corspuscles. Scale bars in all images represent 50µm. Images are representative of results obtained in $N \geq 3$ tissue samples per Notch receptor.

We have therefore addressed intrathymic Notch receptor expression in the human thymus and have found, as expected, that Notch1 is broadly expressed (Fig. 28A). PanCK staining revealed that Notch1-expressing cells include cTECs and mTECs (Fig. 28B, C). Additionally, a cortical population of thymocytes, which may correspond to DN cells (Felli et al., 1999) (Van de Walle et al., 2009) (Gonzalez-Garcia et al., 2012) showed Notch1 membrane staining. Notch3 was found expressed by cortical thymocytes in higher proportions than Notch1 (Fig 28D, E). At the medulla, only mTECs expressed Notch3, confirming that a putative role for Notch3 in thymopoiesis is restricted to early developmental stages. Regarding Notch4, its expression was essentially restricted to the medulla, while only rare and scattered thymocytes expressed Notch4 at the cortex (Fig. 28G, H). Importantly, the frequency of those Notch4+ thymocytes increases at the CMJ (not quantified). Importantly, medullary Notch4 expression was mainly restricted to thymocytes (Fig. 28I), while Notch4 expression in TECs is quite rare. **In summary, as demonstrated for Notch ligands, Notch receptors are differentially distributed in specific thymic niches and cell types.**

1.5. Notch1 signaling is active in cortical thymocytes and CD34+ intrathymic progenitors of both human and murine thymus.

Although giving valuable hints about putative Notch signaling at particular thymic niches, neither Notch ligand nor Notch receptor expression ensures that Notch activation is formally occurring *in vivo* in those thymic locations. Aware of this limitation, we used a monoclonal antibody that specifically recognizes the intracellular domain of Notch1 (ICN1) to assess the activation *in vivo* of the Notch1 pathway in the thymus. ICN1 staining revealed a broad and quite homogeneous pattern of active Noth1 expression across the thymic cortex, while the medulla exhibited lower frequency of ICN1+ cells (Fig. 29A). Because of the importance of Notch1 function in cortical events, we analysed in detail the three cortical microenvironments: SCC, IC and CMJ in different human postnatal thymus samples ($N = 3$), from which $N \geq 15$ images (25x) were acquired per sample. In every region, the exclusion from panCK staining showed that cortical ICN1+ cells are exclusively thymocytes, except at the SCC where some ICN1+ subcapsular cTECs where located under the thymic capsule (Fig. 29B, C, D). When quantifiying ICN1+ cells on each thymic region, we found a highly significant enrichment of ICN1+ cells at the CMJ and at the SCC (Fig. 29E). Similar results were obtained when analysing ICN1 expression in murine thymus (data not shown). Notably, Notch signalling is required for T cell commitment and β-selection, events that occur at the CMJ and the SCC, respectively (Petrie and Zuniga-Pflucker, 2007). It is also worth mentioning that the percentage of ICN1+ cells at the cortex was rather low, ranging from 5 to 15%, depending on the cortical thymic region (data not shown), which suggests that Notch1 is active *in vivo* in a narrow population of cortical thymocytes undergoing the above-mentioned processes. In order to characterize these ICN1-expressing cortical cells, we used the hematopoietic progenitor cell marker CD34 (Fig. 30), as Notch1 must be active in thymus seeding CD34+ progenitors. While it has been described that c-kit+ thymic hematopoietic progenitors in mice enter the thymus through the CMJ where they briefly stay until T cell commitment is induced (Lind et al., 2001) (Witt and Robbins, 2005), no data have been reported to date on the *in vivo* location of human CD34+ ETPs. Our CD34 immunohistochemical stainings on human thymus confirmed that an important proportion of CD34+ progenitors are located at the CMJ, but also at the IC and the SCC (Fig. 30B, C, D), suggesting that CD34+ progenitors rapidly move up to the outer cortex. Importantly, every CD34+ cell located at the thymic cortex in whatever cortical region, SCC, IC or CMJ, expressed active Notch1 (ICN1) (Fig. 31A, B, C), this confirming the capital role that Notch1 plays in early human T cell development. **In summary, our data show that Notch1 receptor is active *in vivo* in human CD34+ progenitors, as well as in a narrow population of CD34- cortical cells, confirming the *in vivo* implication of Notch1 signalling in early human T cell development**.

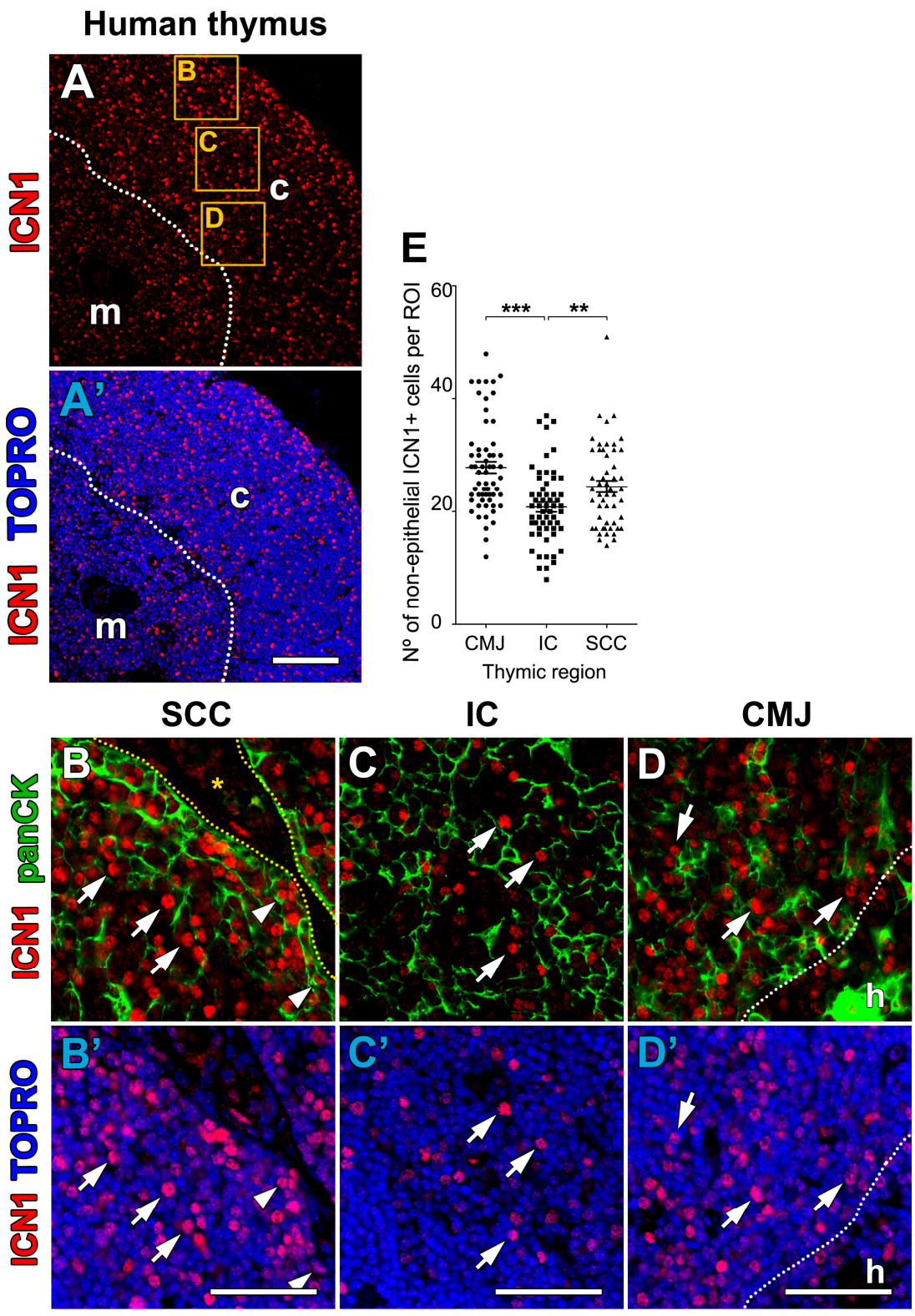


Figure 29. Notch1 is active in cortical thymocytes that accumulate at the SCC and CMJ. Images show FFPE sections of human postnatal thymic tissue (< 17 months-old) stained for the intracellular active form of Notch1 (ICN1) (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A,* General view of active Notch1 distribution in the human thymus. Thymic regions are labeled as *c:* cortex; and *m:* medulla. *Dotted line:* CMJ. Scale bar: 100µm. *B, C, D*, Detailed expression of ICN1 in the three main regions of the thymic cortex: SCC, IC and CMJ, respectively. Total number of ICN1+ cells in each region (E) was quantified by defining niche-specific ROIs (*B, C, D*, yellow squares; $N \geq 45$) obtained from 25x images. *h:* Hassal's corspuscles. Scale bars: 50µm. Images shown are representative of results obtained from $N \geq 3$ different tissue samples.

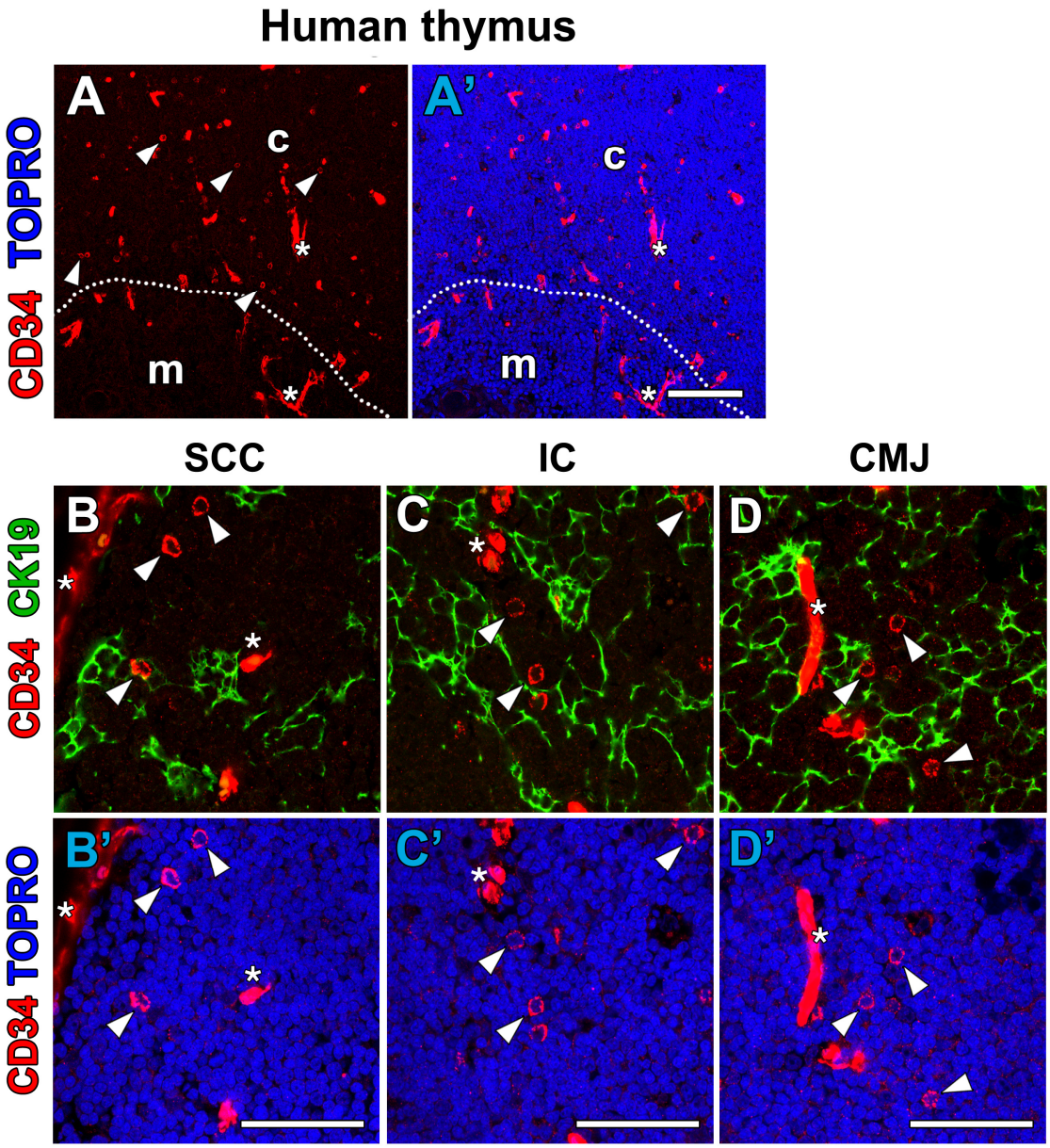


Figure 30. *In vivo* distribution of CD34+ progenitor cells in the human postnatal thymus. Images show FFPE sections of human postnatal thymic tissue (< 17 months-old) stained for CD34 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A,* General view of CD34 distribution in the human thymus. CD34 expression in hematopoietic progenitors (arrowheads) and mesenchymal tissue and endothelium (asterisks) is shown. Thymic regions are labeled as *c:* cortex; and *m:* medulla. *Dotted line:* CMJ. Scale bar: 100µm. *B, C*, *D*, Detailed expression of CD34 in the three main regions of the thymic cortex: SCC, IC and CMJ, respectively. Arrowheads indicate CD34+ progenitor cells; asterisks show CD34 staining in endothelium and/or mesenchymal cells. Scale bars: 50µm. Images shown are representative of results obtained in $N \geq 3$ different tissue samples. **, $p < 0,01$; ***, $p < 0,001$.

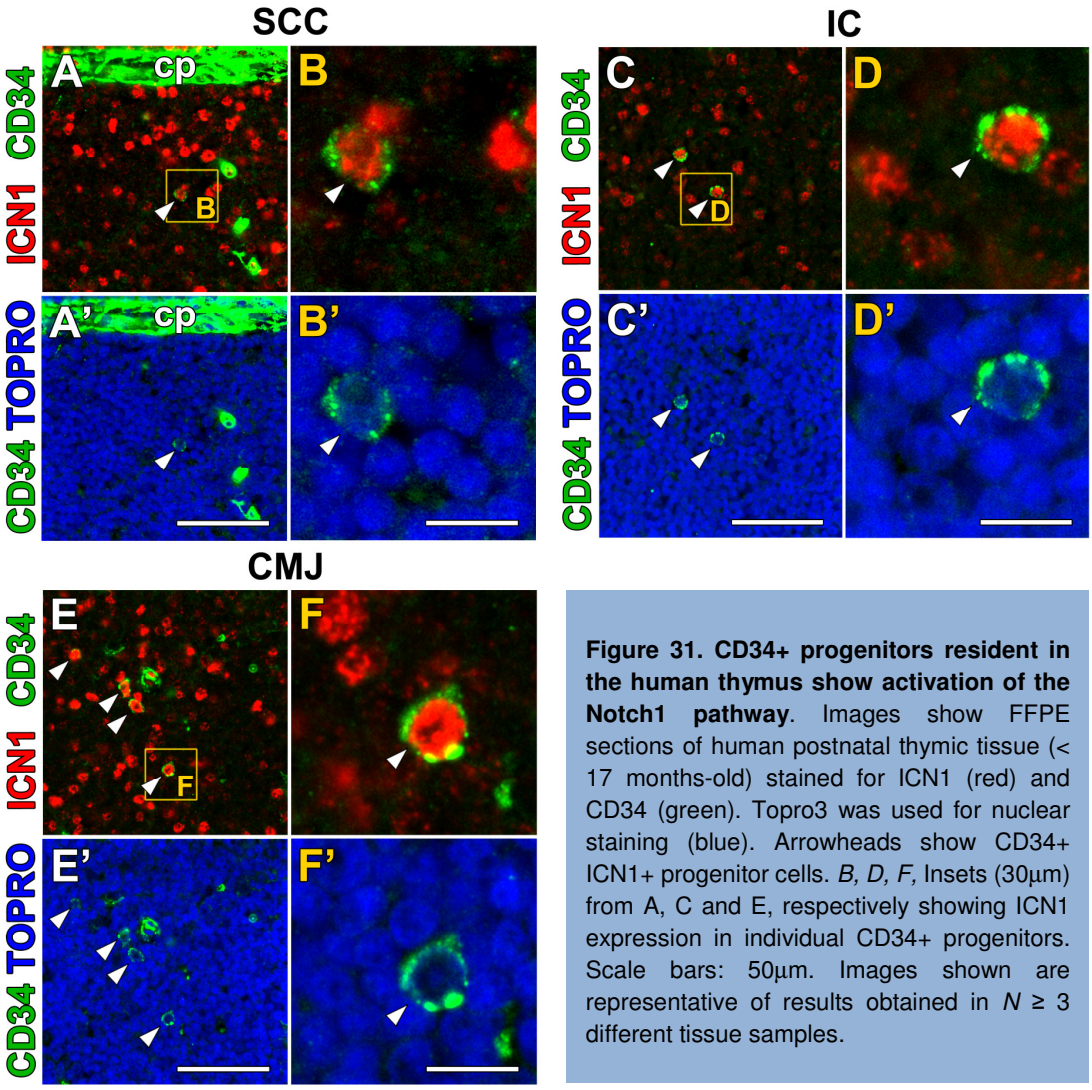


Figure 31. CD34+ progenitors resident in the human thymus show activation of the Notch1 pathway. Images show FFPE sections of human postnatal thymic tissue (< 17 months-old) stained for ICN1 (red) and CD34 (green). Topro3 was used for nuclear staining (blue). Arrowheads show CD34+ ICN1+ progenitor cells. *B, D, F,* Insets (30µm) from A, C and E, respectively showing ICN1 expression in individual CD34+ progenitors. Scale bars: 50µm. Images shown are representative of results obtained in $N \geq 3$ different tissue samples.

II. DIFFERENTIAL ROLE OF NOTCH LIGANDS IN αβ VERSUS γδ T CELL DEVELOPMENT.

The characterization of Notch ligand and receptor distribution *in vivo* in mouse and human thymus has revealed their organization in different thymic niches in which their expression is tightly regulated during ontogeny and involution; this suggesting differential roles for individual Notch ligands in T cell development. In order to shed light on the particular role of individual ligands in T cell development, we performed functional *in vitro* assays using the Notch ligand-transduced OP9 co-culture system (Schmitt and Zuniga-Pflucker, 2002) (De Smedt et al., 2004). The progenitors used in this assay were intrathymic lymphoid-committed progenitors (LP), equivalent to mouse DN2 thymocytes, and characterized as CD34+ CD44lo CD1a- CD4- CD8- cells (Fig. 32A, B). These progenitors have essentially lost their potential to generate myeloid cells, but retain some NK cell potential, which cannot be revealed in the OP9 assay in the absence of exogenous IL-2 or IL-15 (Marquez et al., 1995) (de Yebenes et al., 2002). Therefore, the system is optimal to assess the αβ versus γδ potential and developmental behaviour of common intrathymic progenitors (Ciofani et al., 2006) in response to different Notch ligands.

To this end, we generated five OP9 cell lines (OP9-Dll1, OP9-Dll4, OP9-Jag1, OP9-Jag2 and OP9-GFP) expressing high and homogeneous levels of each ligand, or GFP alone as control. Ligands expression levels were quantitated by MFI of the GFP tracer (Fig. 32C), as discussed above, only equivalent levels of expression may reveal intrinsic Notch signaling features of each particular ligand, as different Notch signalling intensities delivered *in vitro* generate different T-cell developmental outcomes (Van de Walle et al., 2009) (Ciofani et al., 2006). Formal proof of ectopic Notch ligand expression by OP9 cells was provided by Western blot (Fig. 32D). As expected from a bicistronic vector, GFP expression levels correlated with protein expression of each Notch ligand. Additionally, all ligands were expressed at the plasma membrane of OP9 cells, as assessed by immunocytochemistry (data not shown). As we have previously shown that Notch1 controls early T cell development by directly regulating *IL7R* expression (Gonzalez-Garcia et al., 2009), we took advantage of this property to directly evaluate the functional capability of the human Notch ligands ectopically expressed on OP9 cells. As shown in Fig. 32E, we found that all four Notch ligand-expressing OP9 cell lines were able to maintain IL7R expression on human intrathymic progenitors developing *in vitro*, although Jag1 was less efficient than the others especially at late time-points (day 25-28), suggesting the induction of lower Notch activation levels. In the absence of Notch ligand (OP9-GFP) however, DN2 progenitors underwent a rapid downregulation of IL7R. **Altogether these**

data indicate that Notch ligand-expressing OP9 stromal cells are able to induce functional Notch signaling in CD34+ CD44^{lo} CD1a- human intrathymic DN2-like progenitors.

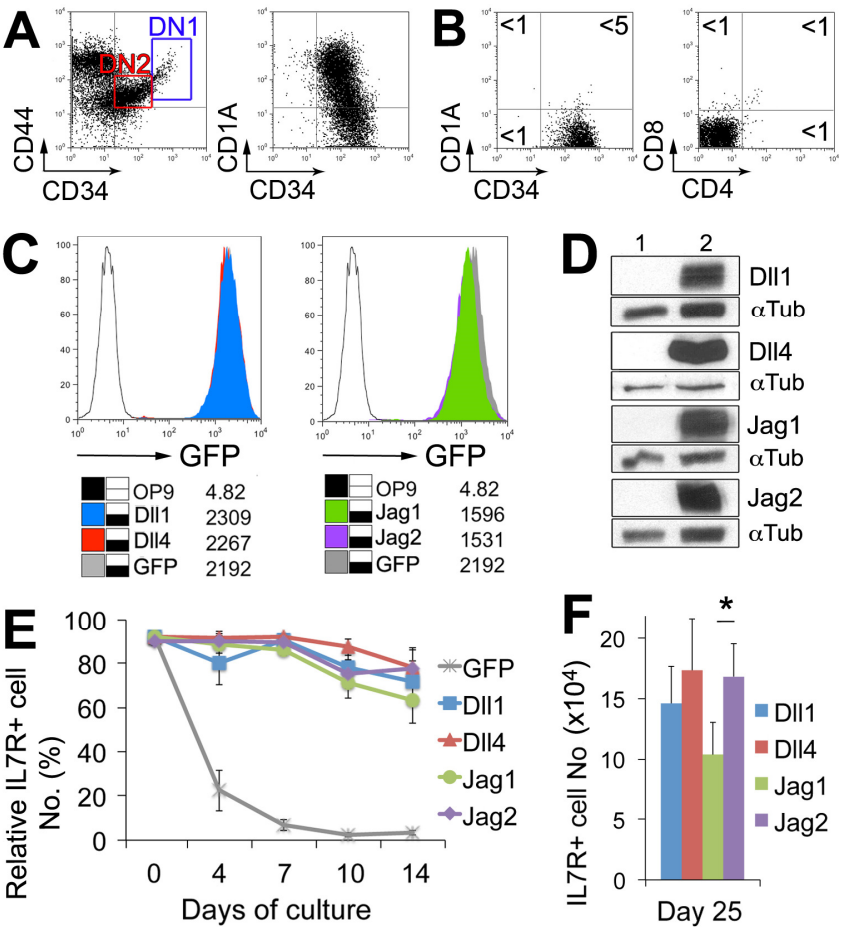
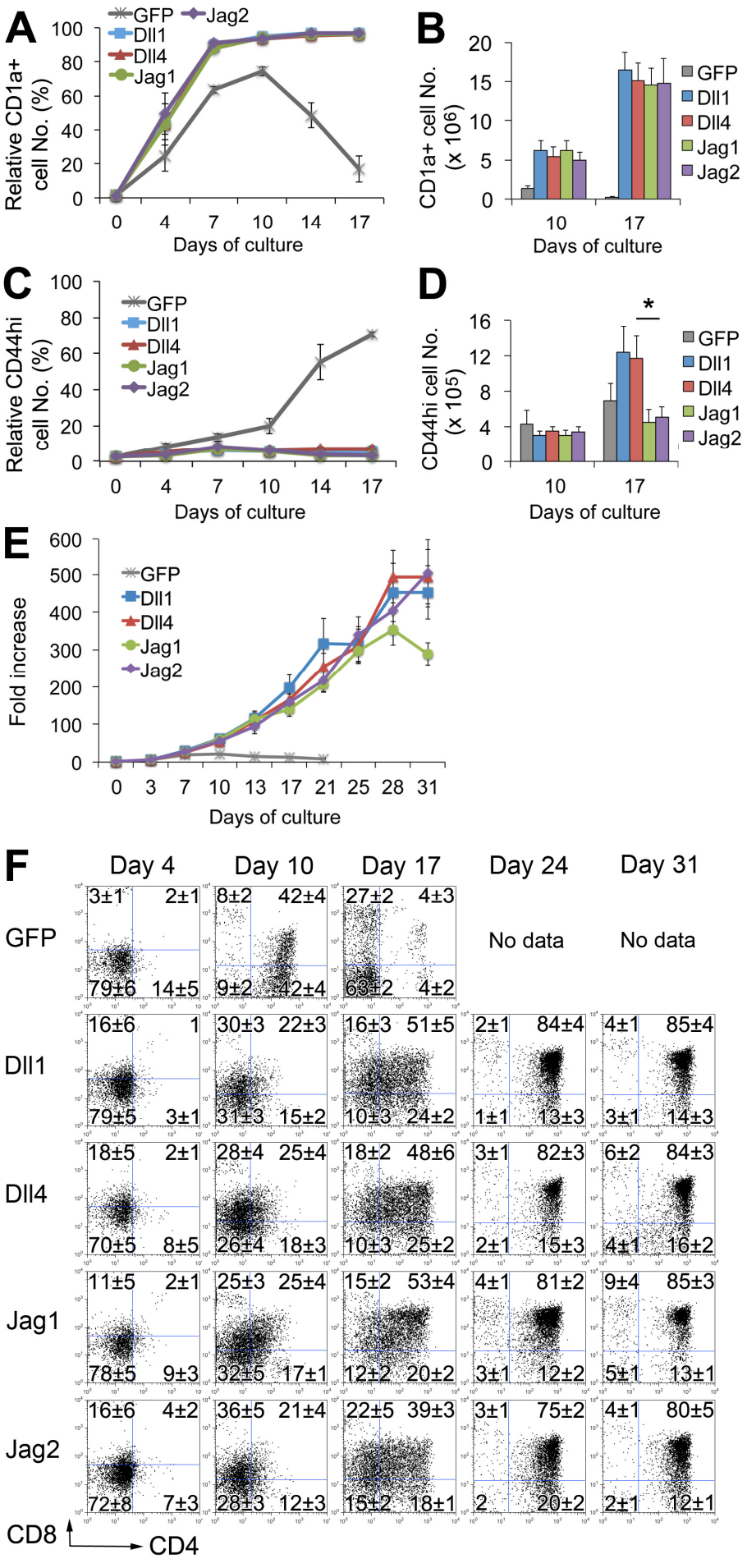


Figure 32. Generation and functional characterization of OP9 stromal cells expressing different human Notch ligands. *A,* Flow cytometry analysis of CD34 and CD44 expression in human thymus cell suspensions depleted of mature thymocytes by sheep red blood cell rosetting (*left*) and further enritched in CD34+ progenitors (*right*) by immunomagnetic cell sorting using anti-CD34-labeled beads. Electronic gates of DN1-like (CD34hi CD44hi) and DN2-like (CD34+ CD44+) thymocyte progenitors are shown (*left*). *B,* Phenotype of DN2-like progenitors isolated from CD34+ thymocytes after depletion of CD1a. *C*, Characterization by flow cytometry of parental OP9 stromal cells and OP9 cells retrovirally-transduced with GFP as control (OP9-GFP) o with distinct human Notch ligands (OP9-Dll1, OP9-Dll4, OP9-Jag1 or OP9-Jag2). Histograms show levels of Notch ligand expression quantified by MFI of the GFP cell tracer. *D,* Western blot analysis of control OP9-GFP (lane 1) and OP9 expressing human Dll1, Dll4, Jag1 or Jag2 Notch ligands (lane 2), with the indicated antibodies. αTubulin expression was used as protein loading control. *E,* Kinetics of IL7R expression in DN2-like human thymocytes co-cultured onto the indicated OP9 stromal cells. *F,* Absolute numbers of IL7R+ cells recovered by day 25 from co-cultures in (E). *; $p < 0,05$. Data are represented as mean +/- SEM of total cell numbers in 5 - 6 independent experiments.

2.1. Distinct Notch ligands induce T-cell commitment and developmental progression of human DN2-like progenitors to the DP stage with similar efficiencies.

As expected, T cell development of CD34+ CD44lo CD1a- progenitors was triggered *in vitro* in the OP9 assay by exposure to Notch ligands. T-cell committed CD1a+ cells were detected as soon as at day 4 of culture. Thereafter, the frequency of CD1a-expressing cells increased rapidly up to 100% during the first week of culture. On the contrary, development of CD1a+ cells committed to the T cell lineage was greatly impaired in OP9-GFP stroma co-cultures, where relative numbers of CD1a+ cells could reach up to 60% by day 10 and declined rapidly thereafter (Fig. 33A). However, absolute numbers were significantly decreased in control cultures, according to an impaired generation and maintenance of CD1a+ T-committed cells when Notch signalling is absent (Fig. 33B). Nonetheless, differentiation from DN2-like progenitors towards the CD4+CD8+ DP stage could occur independently of Notch signaling as reported previously (Van de Walle et al., 2009). The decreased efficiency of T cell generation in OP9-GFP co-cultures paralleled a relative increase in the generation of non-T cell populations, likely corresponding to NK cells (data not shown), defined by high expression of CD44 (Fig. 33C) and whose absolute numbers were specifically increased in Dll1- or Dll4-expressing OP9 cell cultures (Fig. 33D). Cell proliferation studies revealed that numbers of DN2-like progenitors co-cultured on OP9 cells expressing any Notch ligand, increased exponentially during the first three weeks of culture (Fig. 33E). No significant differences were observed on the expansion potential supported by the different ligands, although cell numbers reached up to a 500-fold expansion with Dll1, Dll4 and Jag2 ligands, but only up to a 350-fold expansion in OP9-Jag1 cultures, where total cell numbers reached a plateau after the initial 3-weeks of culture, and thereafter cells declined steadily. In contrast, DN2-like progenitors seeded on OP9-GFP cultures were unable to proliferate and disappeared after two-weeks of culture, independently of the number of DN2 seeded progenitors.

To further investigate the kinetics of T cell differentiation of DN2-like thymocytes under the influence of different Notch ligands, we performed FACS analysis of the acquisition of CD4 and CD8 differentiation markers every 3 to 4 days of culture (Fig. 33F). No significantly different T cell differentiation kinetics were found in response to different Notch ligands, as all supported maximal generation of DP cells between days 24 and 28, and both cell frequencies (%) and absolute numbers of DN, DP, CD4+ or CD8+ were essentially equivalent (Fig. 33G). Acquisition of CD4 and CD8 occurred faster in OP9-GFP co-cultures, where DP cells reached maximal frequencies between day 7 and day 10 (Fig. 33F), but maturation into TCR-expressing cells was very inefficient (see below).


A
Relative CD1a+ cell No. (%)
GFP
Jag2
Dll1
Dll4
Jag1
Days of culture
B
CD1a+ cell No. (x 10⁶)
GFP
Dll1
Dll4
Jag1
Jag2
Days of culture
C
Relative CD44hi cell No. (%)
Days of culture
D
CD44hi cell No. (x 10⁵)
*
Days of culture
E
Fold increase
Days of culture
F
Day 4
Day 10
Day 17
Day 24
Day 31
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Jag2
No data
CD8
CD4

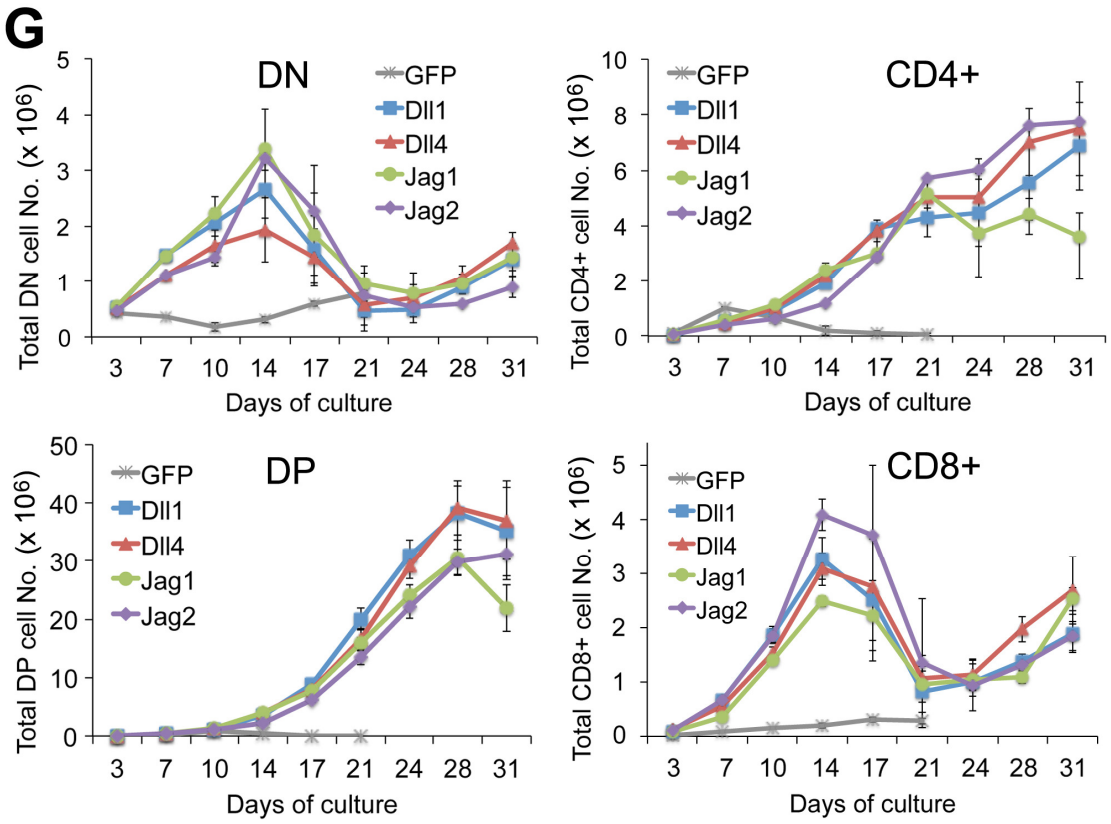


Figure 33. Notch ligand-induced *in vitro* proliferation and T cell commitment of DN2-like human intrathymic progenitors. *A, C,* Kinetic of generation of T cell committed (CD1a+) and non-T cell (CD44hi) populations from DN2-like human intrathymic progenitors co-cultured onto the indicated Notch ligand-expressing OP9 cells or control OP9-GFP cells. *B, D,* Absolute numbers of CD1a+ and CD44hi cells recovered by days 10 and day 17 from co-cultures in A and C, respectively. *; $p < 0,05$. *E,* Fold expansion of DN2-like intrathymic progenitors co-cultured onto the indicated OP9 stromal cell lines during 31 days. Data are represented as mean +/- SEM of absolute cell number in 5 - 6 independent experiments. *F,* Generation kinetics of CD4+ CD8+ DP thymocytes from DN2-like progenitors co-cultured onto the indicated OP9 stromal cell lines, analysed by flow cytometry. Numbers in quadrants indicate the mean +/- SEM percentages of CD4-CD8- DN, CD4+CD8+ DP, CD4+ and CD8+ populations. Histograms shown are representative of 5 independent experiments. *G,* Absolute number generation kinetics of DN, DP, CD4+ and CD8+ thymocytes co-cultures shown in (F). Data show mean +/- SEM of total cell numbers or frequencies (cell %) in 5 independent experiments.

Therefore, **these results indicate that human CD34+CD1a- DN2-like uncommitted progenitors can efficiently differentiate into CD1a+ CD4+CD8+ DP thymocytes in response to Notch signaling *in vitro*, although no differential effects in T-cell commitment and developmental kinetics towards the DP stage were found in response to Notch ligand-specific signaling.**

2.2. Stromal cells expressing distinct Notch ligands recapitulate αβ and γδ T cell differentiation *in vitro* with different efficiencies: Jag2 selectively favors γδ T cell generation.

Previous reports support the view that Notch ligands may influence the TCRαβ versus TCRγδ cell fate decision by delivering differential Notch signal strength to developing thymocytes (Kreslavsky et al., 2010) (Ciofani and Zuniga-Pflucker, 2010) (Van de Walle et al., 2013). In humans, strong Notch signaling promotes TCRγδ development, while attenuation of Notch signal is required for αβ T cell generation *in vitro* (Garcia-Peydro et al., 2003) (Van de Walle et al., 2009). Therefore, we took advantage of the OP9 co-culture assay to directly assess the role of each ligand on αβ and γδ T cell generation from human DN2-like uncommitted progenitors. Results from co-cultures followed during 31 days indicated that all Notch ligands are able to generate both αβ and γδ T cells *in vitro,* although with different efficiencies (Fig. 34). In all cultures, onset of γδ T cell generation preceded αβ T cell onset and was observed as early as at day 10 of culture, although it was not until day 13 when a significant population of γδ cells became evident. Conversely, αβ T cell numbers appeared one to two weeks later (day 17-24), depending on the ligand (Fig. 34A). Interestingly, all Notch ligands, but Jag1 that was equally efficient at supporting differentiation of both lineages, were significantly more efficient supporters of γδ than αβ T cell development (Fig. 34B). Indeed, differentiation kinetics showed that Dll1, Dll4 and Jag2 favored the emergence of higher numbers of γδ cells in both absolute (Fig. 34A) and relative (Fig. 34C, D) terms along culture. An intriguing result was that the efficiency of αβ T cell development in Jag1 cultures was similar to that observed in the presence of Dll1 or Dll4, while emergence of γδ cells was clearly impaired in Jag1 cultures compared to the others. In striking contrast, the other Jagged family ligand, Jag2, was the most efficient inducer/supporter of γδ T cell development, while it was essentially impairing αβ T cell development. Therefore Jag2-triggered signaling almost exclusively thrust differentiation of DN2-like human progenitors towards the γδ T cell lineage (Fig. 34B). **In conclusion, these results show a selective role for particular Notch ligands in the development of either αβ or γδ human T cells. In particular, Jag1 and Jag2, although belonging to the same family of Notch ligands, exhibit opposite roles in αβ versus γδ T cell generation.**

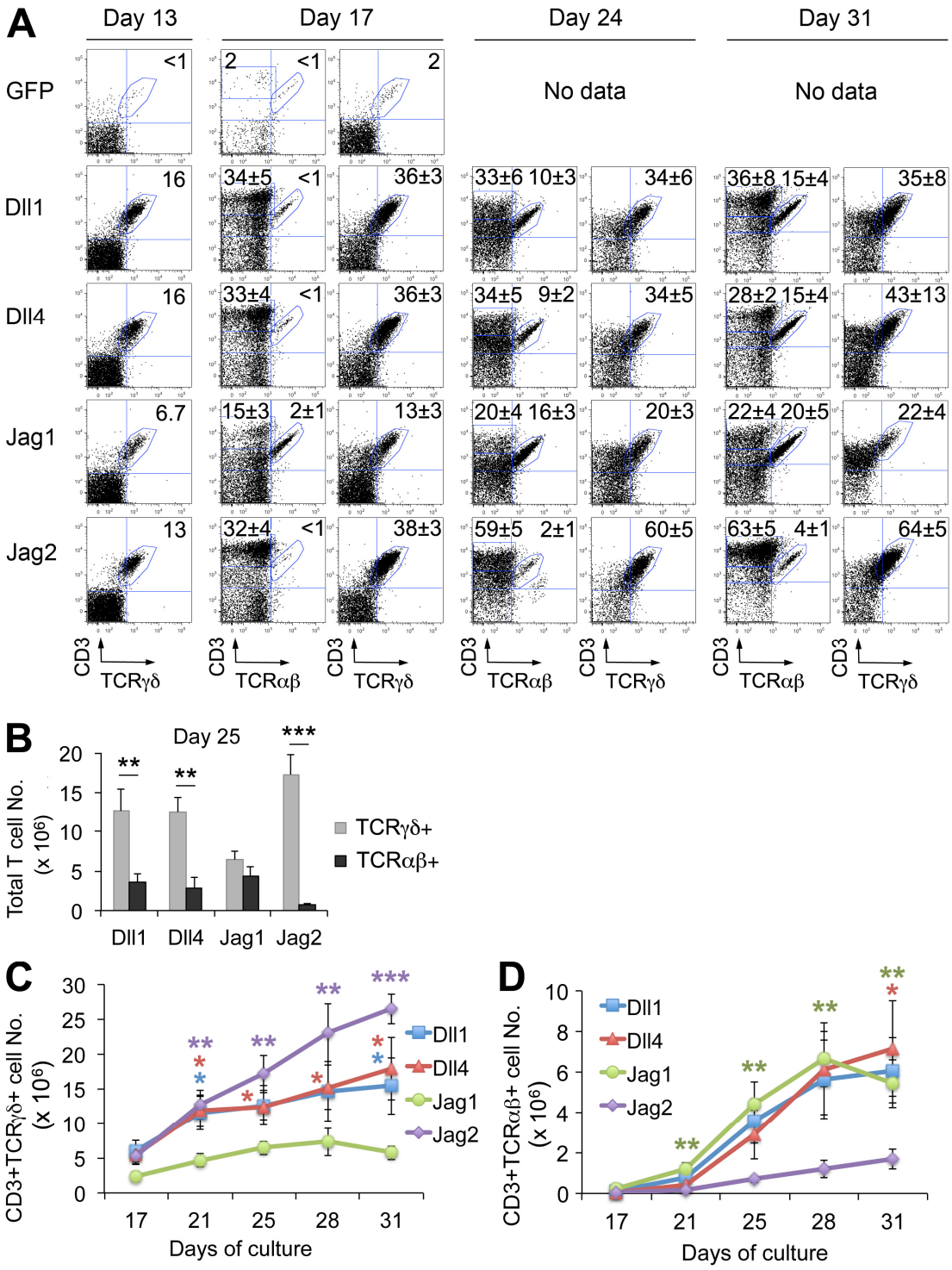


Figure 34. Notch ligand-induced αβ and γδ T cell development from DN2-like human intrathymic progenitors. *A,* Generation kinetics of αβ and γδ thymocytes from DN2-like progenitors co-cultured onto the indicated OP9 stromal cell lines. Numbers in histograms indicate the mean +/- SEM percentages of αβ or γδ T cells. *B,* Absolute cell numbers mean +/- SEM of γδ and αβ T cells recovered at day 25 from the indicated co-cultures. *C, D*, Absolute number generation kinetics of γδ and αβ T cells, respectively, in Notch-ligand OP9 co-cultures. Statistical significance is color-coded relative to Jag1 in *C*, and to Jag2 in *D*. *; $p < 0,05$. **, $p < 0,01$. ***, $p < 0,001$. Data are represented as total cell number mean +/- SEM of five independent experiments.

2.3. Jag2 signaling impairs TCRαβ development at the β-selection checkpoint.

To investigate in more detail the mechanisms underlying the impaired development of αβ T cells promoted by Jag2 signaling, we next analyzed the kinetics of TCR-β chain appearance in developing human progenitors exposed to the different Notch ligands, and focused on intracellular TCRβ (icTCRβ) expression as an unequivocal marker of αβ-lineage commitment. As expected, progenitors co-cultured in the absence of Notch signaling did not acquire TCRβ chain expression, likely because productive rearrangements at the *TCRB* locus were impaired (Wolfer et al., 2002). On the contrary, all four Notch ligands supported the acquisition and expression of a functional TCRβ chain, but with different efficiencies (Fig. 35A). According to the impaired production of γδ T cells in the presence of Jag1, this co-culture showed the highest frequency of icTCRβ+ cells at early time points and all along the whole culture period, but production of icTCRβ+ cells supported by Jag1 was equivalent to that observed in Dll1 and Dll4 cultures in absolute terms (Fig. 35B), this confirming that Dll1, Dll4 and Jag1 are equally efficient at promoting and/or supporting αβ T-cell development. In contrast, cells expressing icTCRβ were significantly reduced in both absolute and relative numbers in Jag2 co-cultures (Fig. 35A, B), in which γδ T-cell development was concurrently increased, suggesting that Jag2 signaling impairs the acquisition of a functional TCRβ chain by common αβ/γδ thymocyte progenitors, but favors the expression of a γδ TCR. Nonetheless, we cannot exclude the possibility that Jag2 affects the survival and expansion of lineage-restricted pre-committed progenitors. **In conclusion, these data indicates that the Notch Jag2 influence γδ and αβ T cell generation *in vitro* by precisely impairing the generation of TCRβ+ αβ-lineage cells.**

2.4. Distinct Notch ligands specify similar gene expression programs in human αβ/γδ common intrathymic progenitors prior to TCR expression.

In order to understand the precise mechanism/s responsible for the differential role of Jag2, and possibly other Notch ligands, on αβ- versus γδ-lineage decision of developing thymocytes, we decided to investigate the nature of the molecular cues that could be responsible for this differential outcome. As pointed out above, signals provided by different Notch ligands could influence αβ versus γδ T cell production in either an inductive or a selective way. In the former situation, specific Notch ligands may act as inductive signals for αβ or γδ cell fate determination prior to TCR expression, while in the latter Notch signaling would just favor the

survival and/or proliferation of cells that have already acquired a functional lineage-restricted TCR (or pre-TCR).

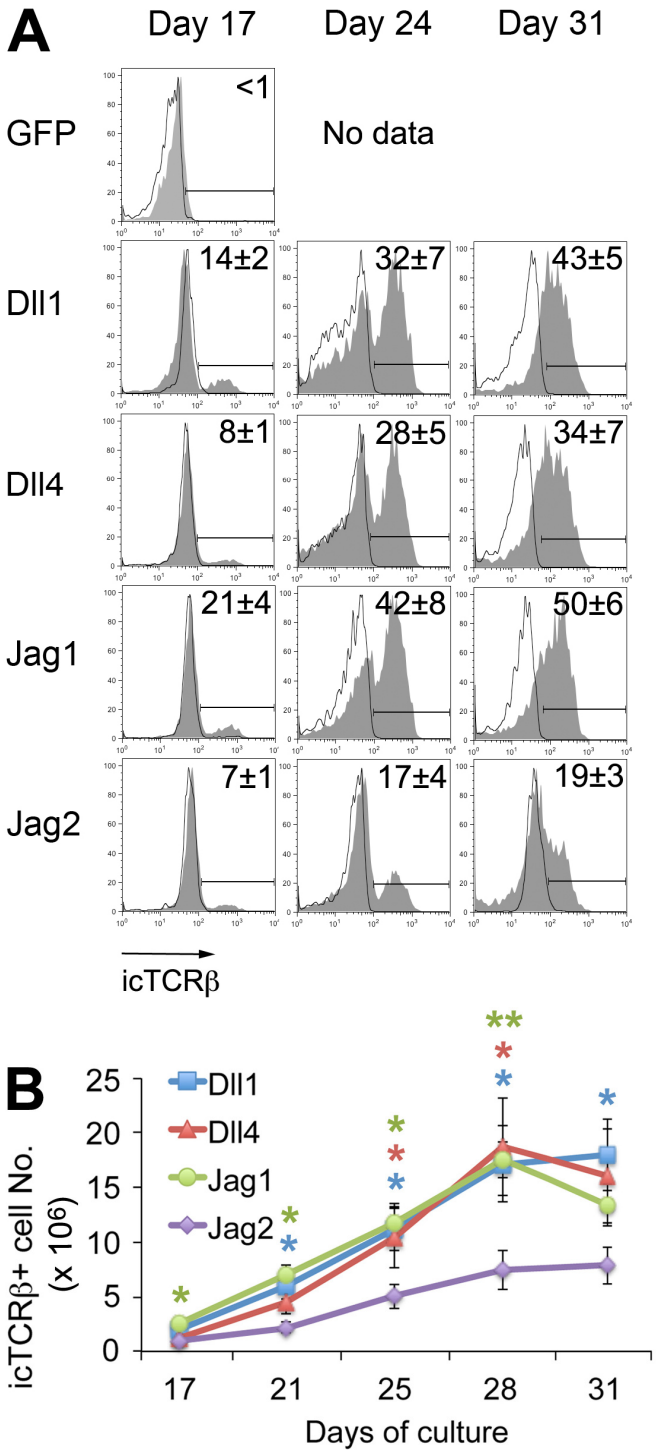
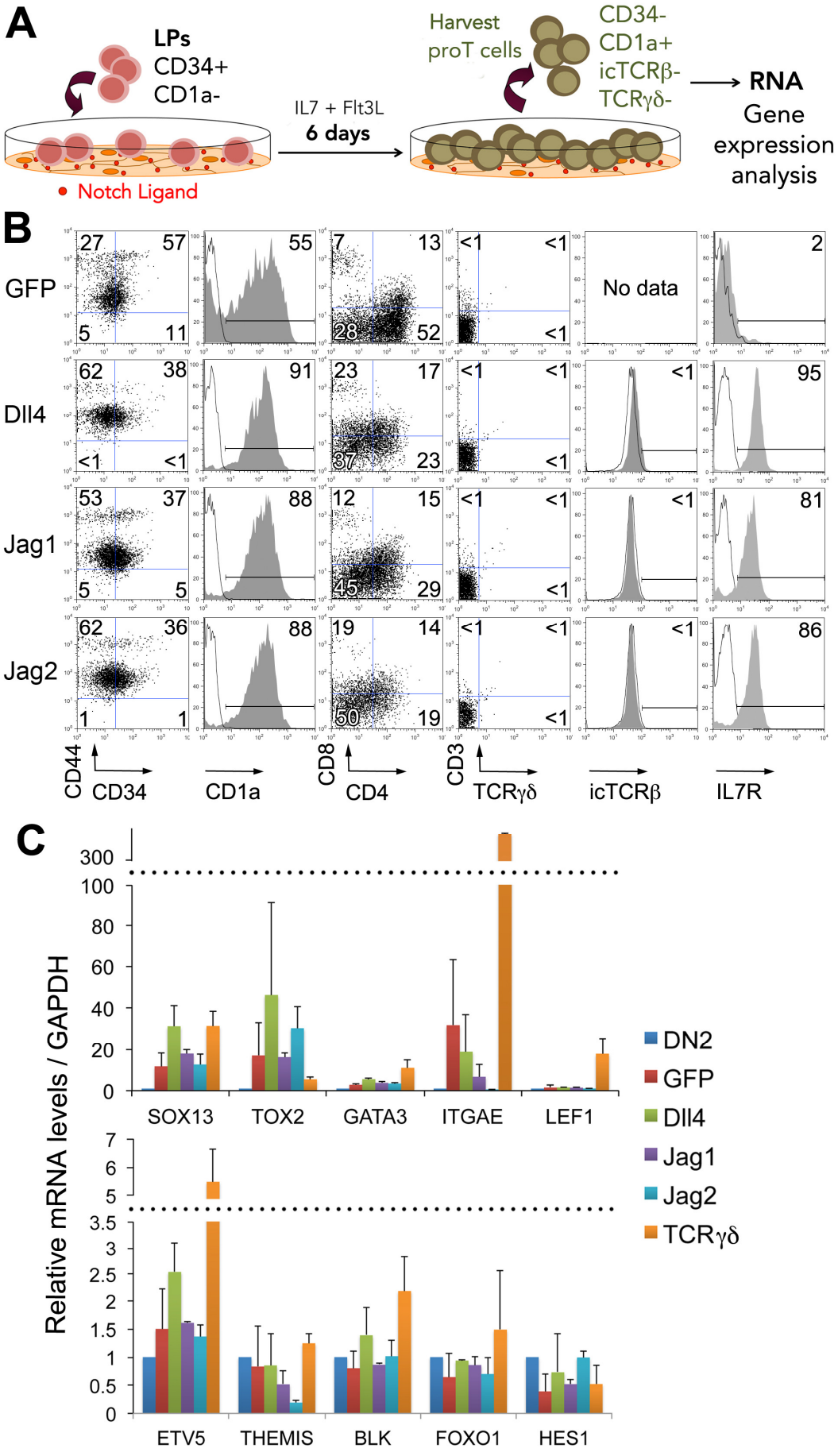


Figure 35. Notch ligand-induced generation of intraceullar TCRβ+ human thymocytes from DN2-like progenitors. *A.* Flow cytometric analysis of intracellular TCRβ expression (icTCRβ) in human DN2-like thymocytes co-cultured during 4 weeks onto OP9 stromal cells expressing the indicated Notch ligands. Numbers in histograms indicate mean +/- SEM cell frequencies (%). Data are representative of 3 independent experiments. *B,* Absolute number generation kinetics of icTCRβ+ cells in co-culture shown in (A). Statistical significance is color-coded relative to Jag2. *; $p < 0,05$. **, $p < 0,01$. ***, $p < 0,001$. Data are represented as mean +/- SEM of 6 independent experiments.

If the first scenario is correct, αβ- and γδ-primed T cells must exhibit an specific transcriptional print before TCR expression, in a similar way that has been described for T lineage-committed cells (Thompson and Zuniga-Pflucker, 2011) (Tabrizifard et al., 2004) (Yui and Rothenberg, 2014). To determine whether T cell progenitors can in fact be transcriptionally

primed towards a particular T cell fate in a Notch ligand-specific manner, we co-cultured yet uncommitted CD34+CD1a- DN2-like progenitors isolated from the human thymus onto OP9-GFP, OP9-Dll4, OP9-Jag1 and OP9-Jag2 stromal cells. Given that differentiation outputs with Dll1 and Dll4 were equivalent, only Dll4 cultures were included in the study. Thymocyte populations emerging from the cultures were recovered by day 6, and RNA was extracted and assayed for αβ/γδ lineage-associated transcription factors (Fig. 36A). At this stage, developing thymocytes are committed to the T cell lineage, as they are CD1a+ and express low levels of CD4 and CD8, but they still lack both expression of particular TCR at the plasma membrane as well as intracellular TCRβ chain expression (Fig. 36B), so putative differential transcriptome profiles would be related to Notch rather than to TCR signaling.

Lineage-specific transcription factors to be analyzed were selected based on the extensive expression data available from the ImmunoGenome project (www.immgen.org). Differential expression analyses between ETPs (preT_ETP_2A_Th) and γδ- (Tgd_Th) and αβ-lineage murine populations (T_DP_Th) revealed a number of genes that are differentially expressed and may represent putative lineage-specific genes. We focused on 55 of the most differentially expressed genes, and selected seven genes with a clear differential expression profile for further analysis including *SOX13, TOX2, ITGAE, ETV5, THEMIS, BLK* and *FOXO1*. Of them, some have been confirmed in the literature as either γδ or αβ-lineage specific, such as *ETV5, SOX3, RUNX3* and *ID3*, or *THEMIS*, respectively (Melichar et al., 2007) (Jojic et al., 2013) (Malhotra et al., 2013) (Mingueneau et al., 2013). In addition, *LEF1* and *GATA3* T-cell lineage genes and the Notch downstream effector *HES1* were included in the quantitative study as positive controls. Gene expression analyses were performed by qPCR on *ex vivo*-isolated DN2-like human thymocytes and their progeny obtained upon co-culture onto OP9 cells transduced with different Notch ligands or with GFP alone for 6 days; γδ T cells isolated from the human thymus were also included in the study for comparison (Fig. 36C). Expectedly, we found that Notch was active *in vivo* in DN2-like thymocytes, as they showed a constitutive expression of Hes1, but this expression declined upon culture in the absence of Notch signaling onto OP9-GFP stromal cells. Interestingly, levels of Notch activation quantitated by *HES1* expression were maintained in Dll4 and Jag2 cultures, but decreased upon co-culture with Jag1-expressing stromal cells to similar levels to those displayed by OP9-GFP co-cultures or *in vivo* γδ thymocytes (Fig. 36C, lower panel).


A
LPs
CD34+
CD1a-
Notch Ligand
IL7 + Flt3L
6 days
Harvest
proT cells
CD34-
CD1a+
icTCRβ-
TCRγδ-
RNA
Gene
expression
analysis
B
GFP
Dll4
Jag1
Jag2
No data
CD44
CD34
CD1a
CD8
CD4
CD3
TCRγδ
icTCRβ
IL7R
C
Relative mRNA levels / GAPDH
SOX13
TOX2
GATA3
ITGAE
LEF1
ETV5
THEMIS
BLK
FOXO1
HES1
DN2
GFP
Dll4
Jag1
Jag2
TCRγδ

<< Figure 36. Gene expression profile of αβ and γδ lineage-specific transcription factors in DN2-like uncommitted intrathymic progenitors primed with different Notch ligands *in vitro*. *A,* Experimental system to asses Notch ligand-induced αβ and γδ-lineage transcriptional priming of DN2-like uncommitted αβ/γδ uncommited progenitors *in vitro*. DN2-like intrathymic lymphoid progenitors isolated from human postnatal thymus as CD34+ CD1a- thymocytes, were co-cultured onto OP9-Dll4, OP9-Jag1 and OP9-Jag2 stroma during 6 days in the presence of rhIL7 and rhFlt3L. After this period, developing thymocytes still lacking αβ or γδ cell surface markers were harvested and phenotyped by FACS prior cell RNA extraction. *B,* Phenotype of DN2-like progenitors co-cultured in (A). Numbers in histograms indicate the frequency of positive cells for the indicated markers. Data shown are representative of 3 independent experiments. *C,* Quantitative RT-PCR analysis of αβ and γδ lineage-specific transcription factors in cells recovered from co-cultures in (A). Data show mRNA expression levels of the indicated transcription factors normalized to GAPDH and relative to values obtained for *ex-vivo* isolated DN2-like progenitors. Data show mean +/- SEM of triplicates from 2 sets of independent samples.

Expression analysis of selected genes associated with αβ or γδ T-cell specification revealed no significant differences between progenitors cultured in the presence of Jag1 or Jag2 ligands, which may represent selective supportive ligands for either αβ-lineage or γδ-lineage T cells, respectively. Even *SOX13* and *ETV5*, which have been described as γδ-lineage specific transcription factors and are expressed at high levels in *ex vivo*-isolated γδ thymocytes (Fig. 36C), were equally expressed in cells co-cultured onto either OP9-Jag1 or -Jag2 stromas; although both genes were significantly increased in OP9-Dll4 cultures. In addition, *TOX2* was induced by Jag2 and Dll4 signaling, while *ITGAE* expression was low in the presence of any Notch ligand, but was highly expressed on primary γδ thymocytes. Therefore, no correlations could be established between a specific transcriptional profile and a particular Notch ligand. **In summary, we can conclude that signals delivered by Notch ligands do not significantly influence the αβ/γδ-specific lineage transcriptional program of the immediate progeny derived from human DN2-like uncommitted progenitors before acquisition of TCR expression.**

2.5. Characterization of human intrathymic γδ T cell developmental stages: CD1a+ and CD1a- γδ T cell subsets are differentially generated upon signaling induced by distinct Notch ligands.

The finding that Notch signaling does not significantly impact the transcriptional regulation of known lineage-specific transcription factors in αβ/γδ common progenitors developing *in vitro,* suggests that the differential cell output induced by distinct Notch ligands might be related to a selective rather than to an instructive ligand-specific function. Particularly, the improved generation of γδ T cells observed in Jag2 cultures would depend on specific Jag2 signals that may promote the survival and/or expansion of γδ-committed, but not αβ-

committed, progenitors at any developmental stage downstream of γδ TCR expression. To test this hypothesis, we next aimed at investigating the particular stage of γδ T cell development at which Jag2 could exert its function, and analyzed in detail the kinetics and phenotype of the γδ T cell progeny emerging *in vitro*. Interestingly, we found that *in vitro* developing γδ T cells were phenotypically diverse considering expression of the CD1a marker (see below). Supporting the physiological relevance of this finding, CD1a is known to define a similar phenotypic heterogeneity among γδ T cells resident *in vivo* in the human thymus, which display three different levels of surface CD1a, from negative to high (Fig. 37A). According to previous data (Offner et al., 1997) (Van Coppernolle et al., 2012), we also found that thymic γδ T cells are heterogeneous in terms of CD4 and CD8 expression. DN and CD4+ represent about 40% and 50% of the whole γδ intrathymic compartment, respectively; while cells with the conventional CD8+ phenotype of peripheral blood γδ T cells, together with unconventional DP γδ T cells, comprise a minor population (Fig 37A). What is more important, high CD1a expression was found in CD4+ and DP thymus-specific subsets; but γδ cells bearing the DN or CD8+ phenotypes of conventional γδ cells were mostly devoid or expressed lower levels of CD1a (Fig. 37C, D). Given that CD1a is a conventional marker of immature DP thymocytes in the αβ T-cell lineage (Galy et al., 1993), these data suggested that CD1a expression could define an equivalent immature intrathymic developmental stage in the human γδ T cell lineage. Therefore, it is possible that CD1a+ γδ thymocytes are the progenitors of CD1a- γδ cells in the human thymus as previously proposed (Van Coppernolle et al., 2012).

Next, we compared the phenotype of human γδ thymocytes resident *in vivo* with that of γδ T cells developing *in vitro,* and found that cells expressing CD1a were significantly more frequent than CD1a- γδ T cells in all culture conditions (Fig. 37E). However, clear differences were observed in OP9-Jag1 cultures compared with the others, as CD1a+ γδ cells were significantly less frequent in the former (Fig. 37E, *left*). Notably, CD1a+ γδ cell were less efficiently generated and their relative number decreased progressively along time in Jag1 cultures; a decrease that paralleled a simultaneous increase in CD1a- γδ T cells that was Jag1-specific (Fig. 37E, right). Therefore, Jag1 seems to be more efficient than other Notch ligands in supporting the development of γδ cells with a mature CD1a- phenotype at the expense of the CD1a+ γδ phenotype.

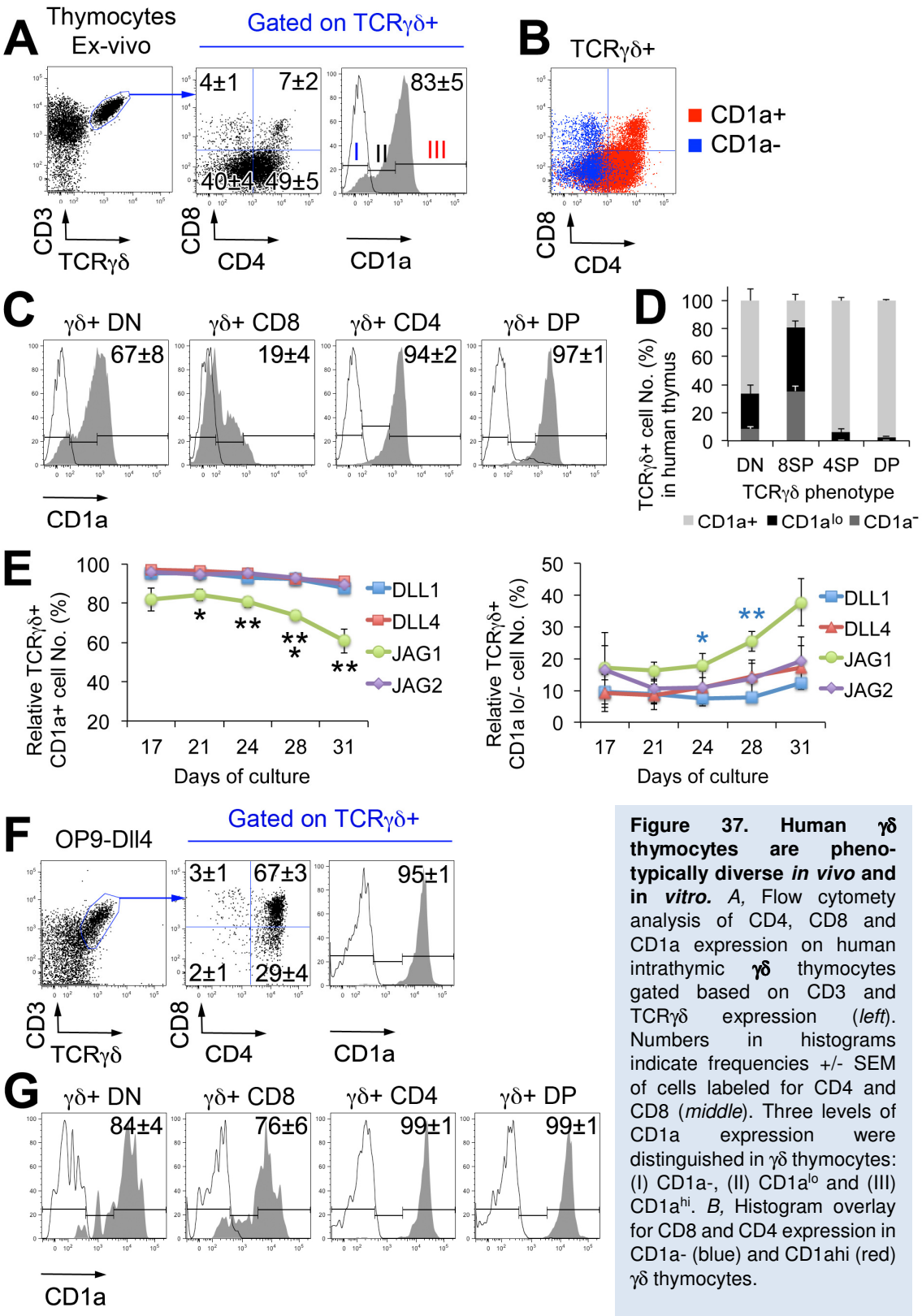
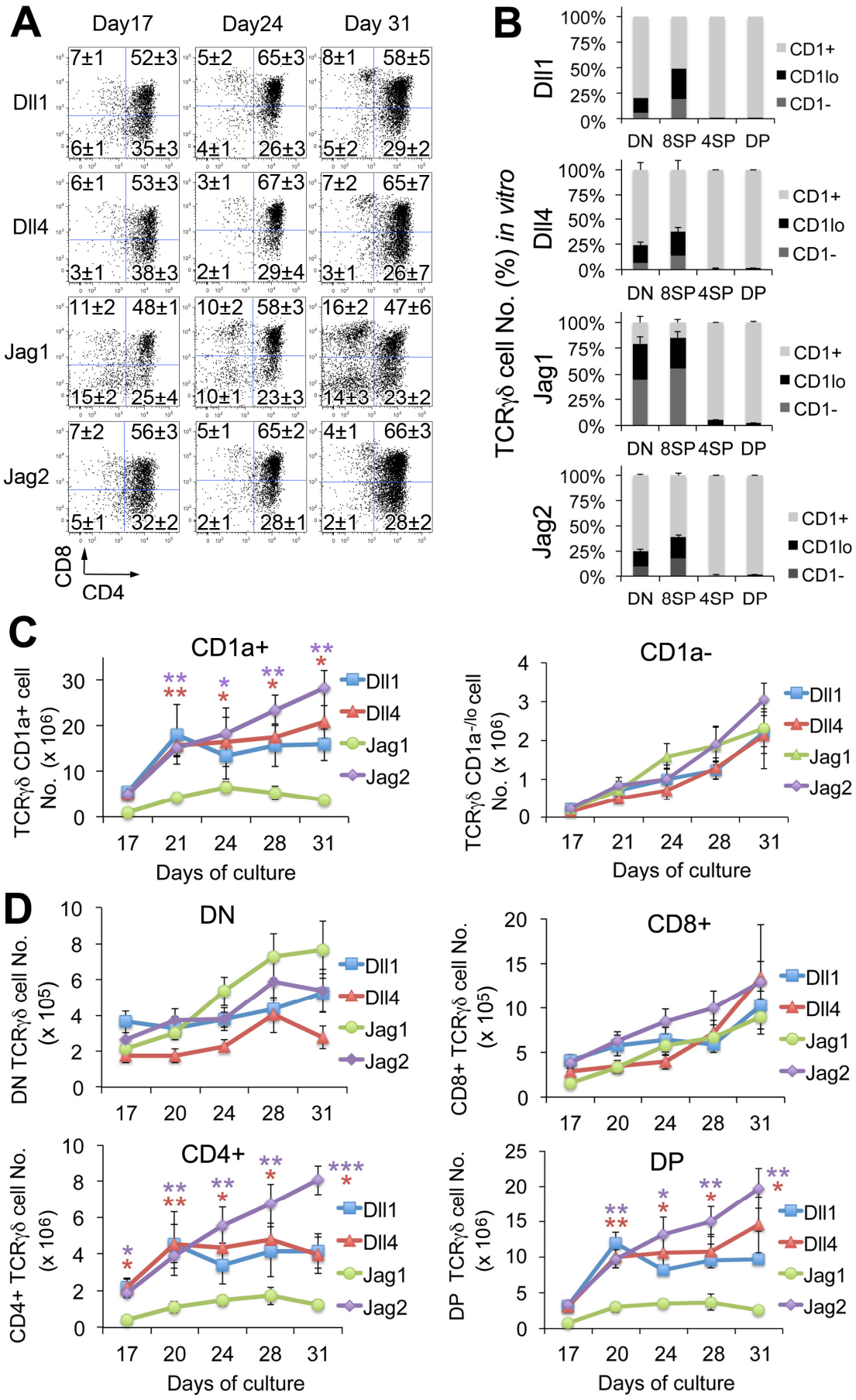


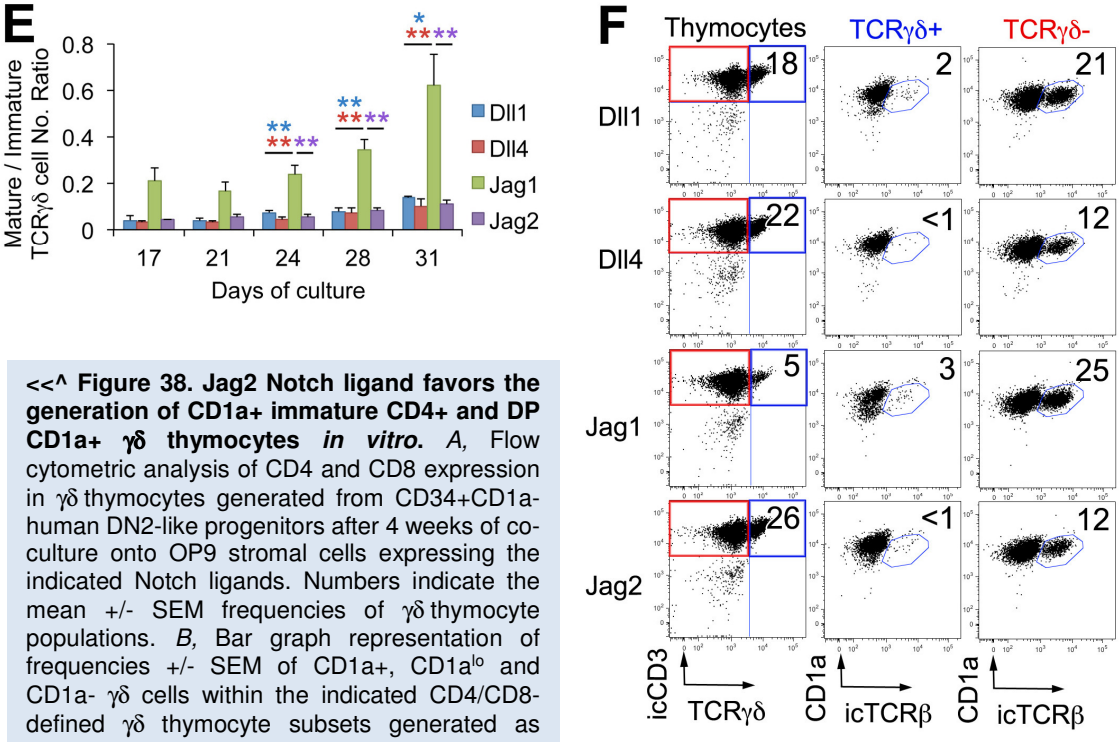
Figure 37. Human γδ thymocytes are phenotypically diverse *in vivo* and in *vitro*. *A,* Flow cytometry analysis of CD4, CD8 and CD1a expression on human intrathymic **γδ** thymocytes gated based on CD3 and TCRγδ expression (*left*). Numbers in histograms indicate frequencies +/- SEM of cells labeled for CD4 and CD8 (*middle*). Three levels of CD1a expression were distinguished in γδ thymocytes: (I) CD1a-, (II) $CD1a^{lo}$ and (III) $CD1a^{hi}$. *B,* Histogram overlay for CD8 and CD4 expression in CD1a- (blue) and CD1ahi (red) γδ thymocytes.

C, Levels of CD1a expression on intrathymic CD4/CD8-defined γδ thymocyte subsets. Numbers in histograms indicate the frequencies +/- SEM of CD1a+ γδ cells representative of 5 independent thymus samples. *D,* Bar graph representation of CD1a+, $CD1a^{lo}$ and $CD1a^{hi}$ cell frequencies within the indicated γδ T cell subpopulation (DN, CD8+, CD4+ and DP). Bars represent mean frequencies +/- SEM of 5 thymus samples. *E,* Generation kinetics of either CD1a+ (left) or CD1a- (*right*) γδ thymocytes derived from DN2-like progenitors in the indicated Notch ligand OP9 co-cultures. *F,* Flow cytometry analysis of γδ cells generated in OP9-Dll4 co-cultures. Numbers in histograms indicate frequencies +/- SEM of 5 experiments. *G,* Levels of CD1a expression on CD4/CD8-defined γδ thymocyte subsets derived in OP9-Dll4 co-cultures. Numbers indicate frequencies +/- SEM of CD1a+ γδ T cells representative of 4 - 5 independent experiments.

To further investigate the developmental pathway followed by γδ T cell progenitors *in vitro*, we next analyzed the expression of CD4 and CD8 markers on the CD1a+ and CD1a- progenies. We found that, like their *in vivo* counterparts, *in vitro*-derived CD1a+ γδ T cells were highly enriched (99 ± 1%) within the DP and CD4+ cell subsets, which represented the major populations derived in culture from DN2-like progenitors (see Fig. 37F, G as an example of γδ T cells generated in OP9-Dll4 cultures). Accordingly, DN γδ thymocytes, which comprise almost half of γδ thymocytes *in vivo*, were misrepresented *in vitro,* and the same was true for γδ cells with the CD8+ phenotype. Notably, poor generation of DN and CD8+ γδ T cells was common in all OP9 cultures, although Jag1 supported the emergence of increased frequencies of both subsets, compared with the other Notch ligands (Fig. 38A). Phenotypic analysis of the major CD1a+ population generated in all culture conditions confirmed that they were DP or CD4+ (Fig. 38B). Interestingly, a major proportion of DN and CD8+ γδ cells generated under Dll1, Dll4 and Jag2 signaling, also expressed the immature CD1a cell marker, suggesting that they were still immature (Fig. 38B). Only in Jag1 cultures DN and CD8+ γδ cells were mostly CD1a- or expressed low CD1a levels, like their *in vivo* counterparts (Fig. 37D). **Therefore, OP9 co-cultures recapitulate the generation of all γδ T cell subsets resident *in vivo* in the human postnatal thymus, but with different frequencies, as generation of cells with the phenotype of mature γδ T cells appeared to be impaired by Notch signaling induced by Dll1, Dll4 or Jag2, and only Jag1 seemed permissive for their generation.**

In order to understand the mechanisms responsible for the differential impact of Notch ligands in the generation of CD1a+ and CD1a- γδ cells, we performed kinetic analysis focusing on absolute numbers of each γδ cell subset. We found similar numbers of γδ T cells with a mature CD1a-/low phenotype in all culture conditions (Fig. 38C), which paralleled the generation of equivalent numbers of DN and CD8+ cells (Fig. 38D, *upper graphs*), although a slightly increased production of DN γδ cells was observed in Jag1 cultures. However, immature CD1a+ γδ cells, including both DP and CD4+ γδ phenotypes, were significantly decreased in absolute terms in Jag1 cultures (Fig. 38C, 38D, *lower graphs*), indicating that Jag1 is less efficient that the other Notch ligands at supporting the differentiation and/or expansion of CD1a+ γδ immature, but not of mature CD1a- γδ T cells. As a result, ratios of mature versus immature γδ T cell production were significantly higher in Jag1 cultures compared with the others, differences that increased along the culture (Fig. 38E). As in mice γδ T cells do not follow a DP pathway, which is characteristic of αβ-lineage thymocytes, we could not exclude that DP and CD4+ γδ T cells might be αβ-lineage thymocytes switched to the γδ-lineage. To investigate this possibility, intracellular expression of the TCRβ chain was analyzed in the γδ T cells generated *in vitro* with the different Notch ligands (Fig. 38F).


A
Day17
Day24
Day 31
Dll1
Dll4
Jag1
Jag2
7±1 52±3
6±1 35±3
5±2 65±3
4±1 26±3
8±1 58±5
5±2 29±2
6±1 53±3
3±1 38±3
3±1 67±3
2±1 29±4
7±2 65±7
3±1 26±7
11±2 48±1
15±2 25±4
10±2 58±3
10±1 23±3
16±2 47±6
14±3 23±2
7±2 56±3
5±1 32±2
5±1 65±2
2±1 28±1
4±1 66±3
2±1 28±2
CD8
CD4
B
TCRγδ cell No. (%) in vitro
100%
75%
50%
25%
0%
CD1+
CD1lo
CD1-
DN 8SP 4SP DP
C
CD1a+
CD1a-
TCRγδ CD1a+ cell No. (x 10⁶)
TCRγδ CD1a-/lo cell No. (x 10⁶)
Dll1
Dll4
Jag1
Jag2
Days of culture
D
DN
CD8+
CD4+
DP
DN TCRγδ cell No. (x 10⁵)
CD8+ TCRγδ cell No. (x 10⁵)
CD4+ TCRγδ cell No. (x 10⁶)
DP TCRγδ cell No. (x 10⁶)



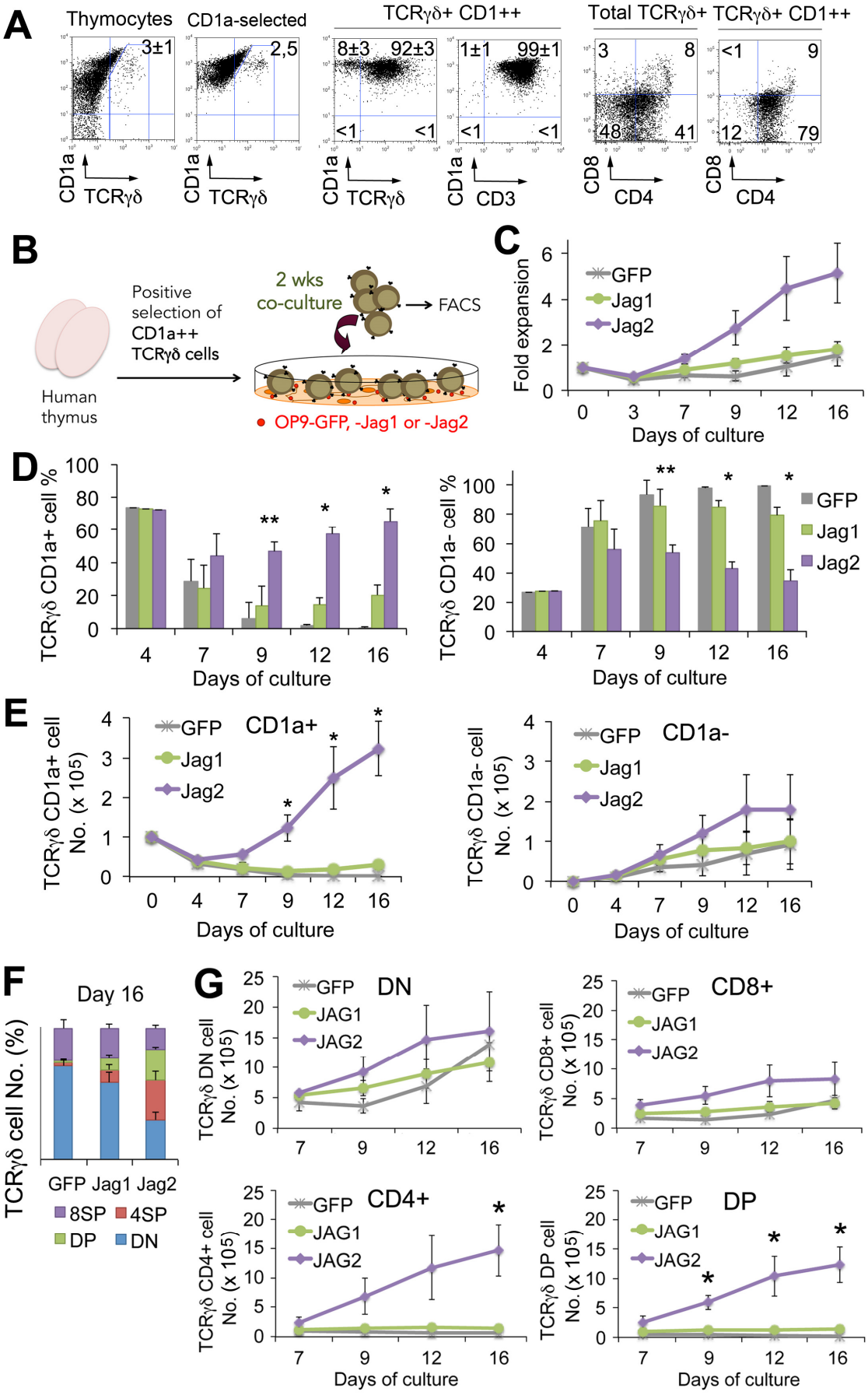
<<^ Figure 38. Jag2 Notch ligand favors the generation of CD1a+ immature CD4+ and DP CD1a+ γδ thymocytes *in vitro*. *A,* Flow cytometric analysis of CD4 and CD8 expression in γδ thymocytes generated from CD34+CD1a- human DN2-like progenitors after 4 weeks of co-culture onto OP9 stromal cells expressing the indicated Notch ligands. Numbers indicate the mean +/- SEM frequencies of γδ thymocyte populations. *B,* Bar graph representation of frequencies +/- SEM of CD1a+, CD1a^{lo} and CD1a- γδ cells within the indicated CD4/CD8-defined γδ thymocyte subsets generated as indicated in (A). *C, D,* Absolute number generation kinetics of gd thymocyte subsets defined by either CD1a+ or CD1a- and CD4 and/or CD8 expression, respectively, recovered from co-cultures shown in (A). Statistical color-code significance relative to Jag1 is shown. *; p < 0,05. **, p < 0,01. ***, p < 0,001. *E,* Bar graph representation of mature CD1alo/CD1a- and immature CD1a+ mean ratios +/- SEM of γδ thymocyte generation in co-cultures shown in (A).**, p < 0,01. ***, p < 0,001. Data shown is representative of 6 independent experiments. *F,* Flow cytometry analysis of icTCRβ in TCRγδ+ cells recovered at day 17 from Notch ligand OP9 co-cultures. Blue and red gates define γδ– and αβ-lineage thymocytes, respectively. Numbers in histograms indicate the frequencies of the indicated populations in a representative experiment of two.

We found no expression of TCRβ chain by any TCRγδ+ cell in any culture condition. Thus CD4+ and DP human γδ developmental stages are true γδ-lineage thymocytes.

In contrast to the impact of Jag1 on γδ T cell development, Jag2 was consistently more efficient than the other ligands, especially Jag1, in supporting the generation of γδ T cells at the immature CD1a+ stage; effect particularly evident for CD4+ cells, although DP cells were also significantly increased (Fig. 38D, *lower graphs*). Production of both CD4+ and DP immature subsets expressing CD1a was also higher in Dll1 and Dll4 than in Jag1 cultures, but they reached a plateu phase at day 20 of culture (Fig. 38D). Conversely, such increased production of CD1a+ gd T cell phenotypes augmented along time in Jag2 cultures, suggesting a selective expansion of immature γδ T cells in response to Jag2 signals. **Therefore, higher production of total γδ T cells observed in Dll1 and Dll4, and mostly in Jag2, compared to Jag1 cultures (Fig. 34C), was essentially due to the highly efficient generation of DP and CD4 immature γδ T cell populations in the former, while Jag1 was more permissive than the other ligands for the generation of mature γδ T cells.**

2.6. Jag2 selectively supports the proliferation of immature CD1a+ γδ T cells.

The above results prompted us to investigate whether the divergent γδ T cell outcome in Jag1 and Jag2 cultures was actually the result of an opposed potential to support the maturation and/or expansion of CD1a+ γδ thymocytes. To this end, we analyzed the differentiation potential of *ex-vivo* isolated human immature γδ thymocytes expressing high levels of CD1a (>99% CD1a++) in OP9 co-cultures. As expected, isolated CD1a+ γδ thymocytes from human thymus were mostly CD4+ (about 80%), and a minor proportion (about 10%) were DP (Fig. 27A, *right*). The resulting 10% left were CD1a+ DN γδ cells, which may correspond with γδ T cell progenitors (Van Coppernolle et al., 2012). Analysis of cells recovered from cultures of OP9 cells expressing either Jag1, Jag2 or no ligand at different time points, revealed that Notch signaling induced by Jag2 resulted in a 5-6-fold cellular expansion, while cell numbers did not significantly increased in OP9-Jag1 and OP9-GFP co-cultures (Fig. 39C). Kinetic analyses revealed the downregulation of CD1a and the generation of CD1a- γδ thymocytes at early time-points in all cultures, indicating that all culture conditions allowed the maturation of CD1a+ thymocytes into CD1a- γδ T cells. But significant differences were observed between Jag1 and Jag2 cultures (Fig. 39D). By day 7, only about 30-40% of γδ cells remained as CD1a+ in OP9-Jag1 co-cultures and OP9-GFP controls (Fig. 39D, *left*), and thereafter CD1a+ γδ cell numbers decreased steadily and essentially disappeared, while CD1a- γδ cells increased concurrently (Fig. 39D, *right*). Conversely, proportions of CD1a+ γδ cells increased significantly from day 7 in Jag2 cultures, which may suggest an impaired survival of mature CD1a- cells developing in the presence of Jag2. In contrast to this possibility, we found that absolute numbers of CD1a- cells arising in OP9-Jag2 cultures were similar those in OP9-Jag1 or OP9-GFP cultures (Fig. 39E), and numbers of γδ cells that displayed the mature DN or CD8+ phenotype were similar as well regardless of the Notch ligand expressed in OP9 cultures (Fig. 39G). Moreover, absolute numbers of CD1a+ γδ cells increased steadily in OP9-Jag2 cultures and this expansion was confirmed to involve immature γδ cells with either the CD4+ or the DP phenotype (Fig. 39E, F). Therefore, Jag2 seems to be highly efficient as an inducer of the proliferative expansion of immature γδ T cells, which however seem to undergo maturation at lower rates than cells developing in the absence of Notch signaling or in the presence of Jag1. Supporting this possibility, mature DN and CD8+ γδ cells represented the major cell output in OP9-GFP and OP9-Jag1 cultures by day 16 (Fig. 39F). However, about one third of γδ cells in OP9-Jag2 cultures still remained as CD4+, and a similar proportion displayed the immature DP phenotype, suggesting an impaired maturation of γδ T cells compared with OP9-Jag1 or control OP9 cultures (Fig. 39F) under Jag2 signaling.


A
Thymocytes
CD1a-selected
TCRγδ+ CD1++
Total TCRγδ+
TCRγδ+ CD1++
3±1
2,5
8±3
92±3
1±1
99±1
3
8
<1
9
48
41
12
79
CD1a
TCRγδ
CD3
CD8
CD4
B
Positive selection of CD1a++ TCRγδ cells
Human thymus
2 wks co-culture
FACS
OP9-GFP, -Jag1 or -Jag2
C
Fold expansion
GFP
Jag1
Jag2
Days of culture
D
TCRγδ CD1a+ cell %
TCRγδ CD1a- cell %
Days of culture
E
TCRγδ CD1a+ cell No. (x 105)
TCRγδ CD1a- cell No. (x 105)
CD1a+
CD1a-
Days of culture
F
Day 16
TCRγδ cell No. (%)
GFP Jag1 Jag2
8SP
4SP
DP
DN
G
TCRγδ DN cell No. (x 105)
TCRγδ CD8+ cell No. (x 105)
TCRγδ CD4+ cell No. (x 105)
TCRγδ DP cell No. (x 105)
DN
CD8+
CD4+
DP
JAG1
JAG2
Days of culture

<<^ Figure 39. Jag2 supports the selective expansion of CD1a+ immature CD4+ and DP γδ T thymocytes resident in the human thymus. *A,* Flow cytometry analysis of CD1a+ γδ cells isolated by magnetic cell sorting from human thymus. Human γδ thymocytes expressing CD1a were isolated by sequential CD1a and TCRγδ magnetic cell sorting. CD8 and CD4 expression on the isolated CD1a+ γδ thymocytes is shown. Numbers in histograms represent mean +/- SEM frequencies on the indicated cell populations. Data shown are representative of 1 - 3 independent experiments. *B,* Experimental design for *in vitro* differentiation of *ex-vivo* isolated CD1a+ γδ**+** human thymocytes co-cultured onto Jag1- or Jag2-transduced OP9 cells. CD1a+ **γ**δ+ human thymocytes isolated as shown in (A) were co-cultured for two weeks onto OP9-GFP, OP9-Jag1 or OP9-Jag2 stromal cells in the presence of rhIL7 and rhFlt3L. *C,* Fold expansion of CD1a+ γδ+ thymocytes co-cultured during 16 days onto the indicated OP9 stromal cells. Data are represented as mean +/- SEM of total cell number in 3 independent experiments. *D,* Kinetics of generation of CD1a+ (*left*) and CD1a- (*right*) gd thymocytes in the indicated Notch ligand OP9 co-cultures. Data are represented as mean +/- SEM frequencies of the indicated cell subsets in 3 independent experiments. *E,* Absolute number generation kinetics of CD1a+ γδ+ (*left*) and CD1a$^{lo/-}$ γδ+ cells (.right) in the indicated Notch ligand OP9 co-cultures. Data show mean +/- SEM absolute numbers in 3 independent experiments. F, Bar graph show mean +/- SEM frequencies of the indicated CD4/CD8-defined γδ subsets generated by day 16 in the indicated co-cultures. *G,* Absolute number generation kinetics of the indicated CD4/CD8-defined γδ subsets generated in the OP9-Jag1, OP9-Jag2 and OP9-GFP co-cultures. Data show absolute cell number +/- SEM of 3 independent experiments. *; $p < 0,05$.

Collectively, these results confirm previous data by Van Coppernolle and coworkers (Van Coppernolle et al., 2012) showing that maturation of CD1a+ γδ thymocytes into CD1a- γδ T cells can efficiently occur upon culture onto OP9 cells lacking ectopic Notch ligands and they extend this finding to OP9-Jag1 co-cultures. But, what is most important, our data also indicate that signaling provided by Jag2 supports a highly proliferative pathway of γδ T cell development involving mostly immature CD1a+ γδ cells of DP and CD4+ phenotype, whose maturation into CD1a- γδ cells is relatively impaired. Therefore, we concluded that **Jag2, and to a lesser extent Dll1 and Dll4 signaling, efficiently supports the generation and expansion of immature CD1a+ γδ T cells, which may require to be released from these Notch ligand signals to stop proliferation and undergo further differentiation into more mature CD1a- γδ T cells. In this scenario, differential expression of Notch ligands at particular niches may critically control the γδ developmental pathway in the human thymus.**

2.7. Vδ repertoire of γδ T cells derived *in vitro* from human thymocyte precursors in response to distinct Notch ligands.

It is known that human Vδ2 γδ T cells are mostly resident in the PB where they display a mature CD1a- DN or CD8+ phenotype. Conversely, Vδ1 γδ T cells represent only 1-2% of PB γδ lymphocytes, but are abundant in the spleen (Falini et al., 1989) and several mucosal tissues (Grossi et al., 1992) (O'Brien et al., 2007) (Hua et al., 2013) and represent the major γδ T cell population in the human thymus (Falini et al., 1989). Analysis of intrathymic Vδ cell subsets showed that most (80%) Vδ1+ γδ thymocytes express CD1a, while Vδ2+ cells are more heterogeneous for CD1a expression (Fig. 40A). Given this heterogeneous distribution of CD1a in γδ cells with distinct Vδ chains, we next assessed whether equivalent populations could be generated *in vitro* in the context of different Notch ligands. To this end, human intrathymic DN2-like progenitors developing onto OP9 cells expressing Dll1, Dll4, Jag1 or Jag2 Notch ligands were analyzed for the expression of Vδ1 and Vδ2, every 3 days along the whole 31 day-culture period. Results revealed that Vδ1 cells were produced more efficiently than Vδ2 in all cultures, but proportions of γδ cells expressing Vδ1, Vδ2, or a different Vδ chain (Leclercq et al., 1993) were essentially similar in all cultures, although Vδ1+ cells were slightly less represented in Jag1 cultures compared to the others (Fig. 40B). According to our results above, absolute numbers of γδ cells expressing any Vδ were considerably reduced in Jag1 cultures compared to the other cultures, confirming an impaired potential of Jag1 to support the development/proliferation of all γδ T cell subsets. Conversely, Jag2 was significantly more efficient than the other ligands in supporting the production an expansion of γδ T cells of the Vδ1+ and Vδ1-Vδ2- subsets, while numbers of Vδ2+ γδ T cells were essentially identical to those emerging in the presence of the other ligands (Fig. 40C). **In summary, these data provide further evidence that Jag2 is the most efficient Notch ligand at supporting the development and expansion of γδ T cell subsets expressing CD1a, such as Vδ1+ cells; while signaling induced by Jag1 appears insufficient to support the expansion of any γδ T cell population, and particularly of Vδ1+ cells, which are mostly CD1a+.**

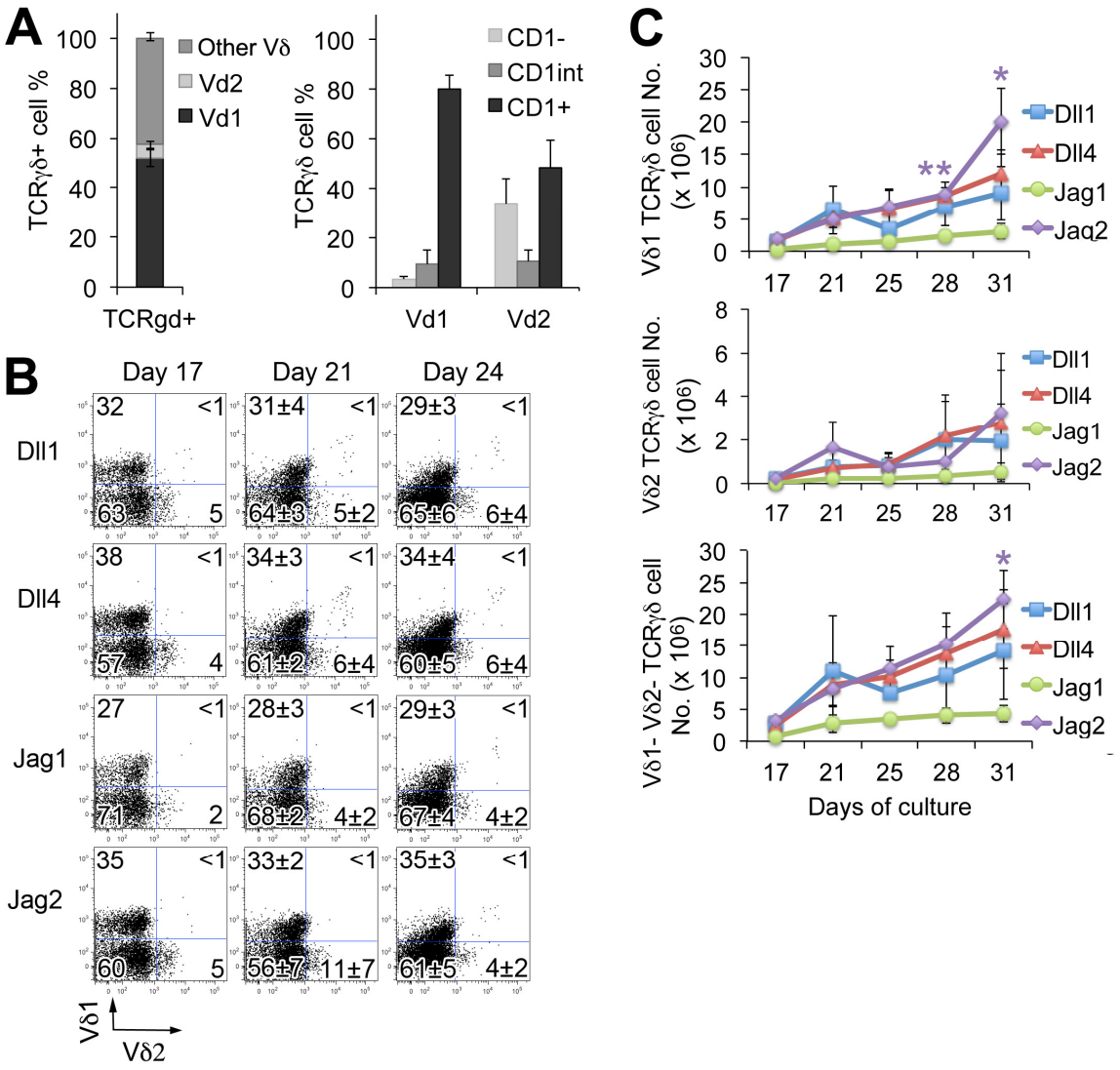


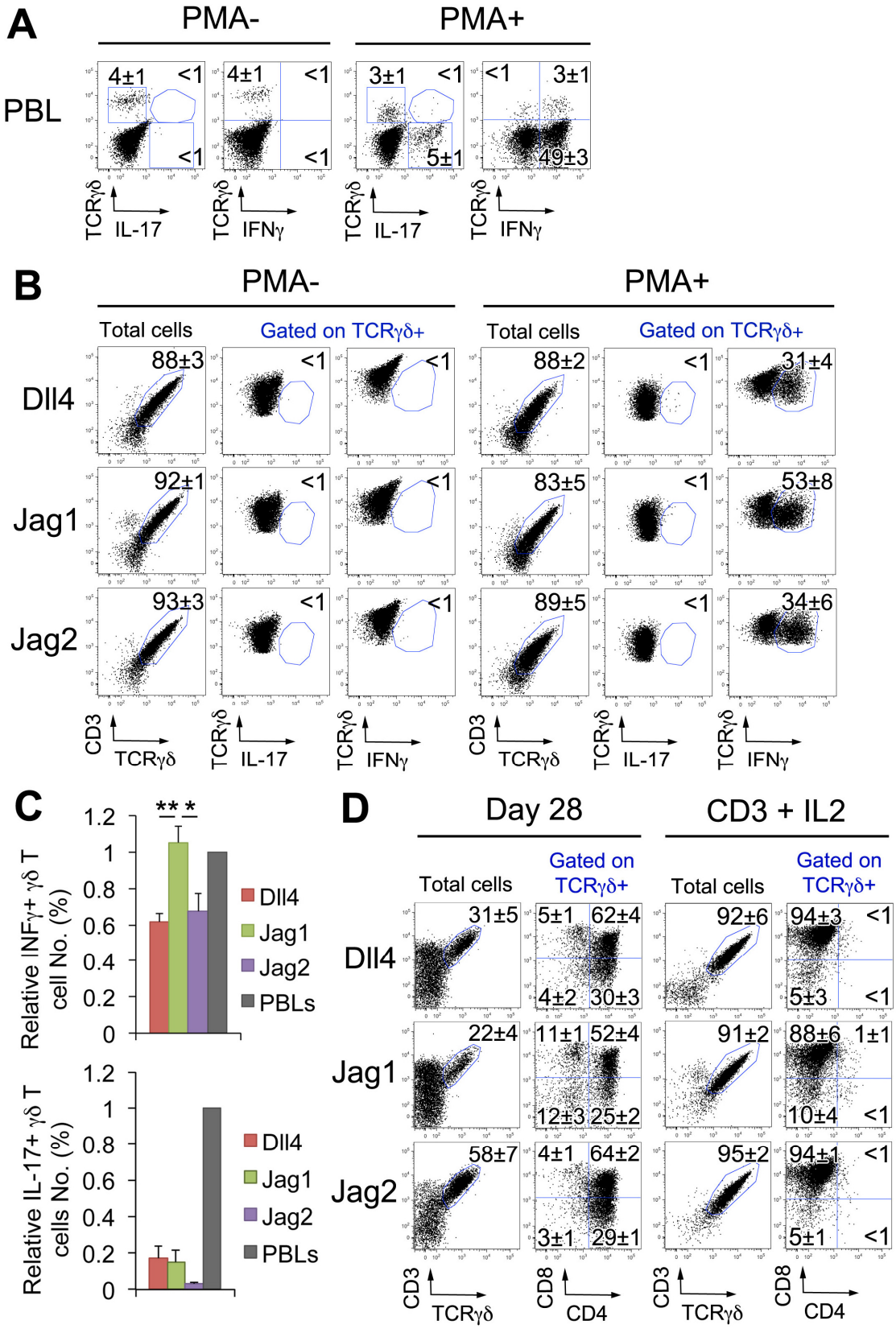
Figure 40. Heterogeneity of Vδ subsets of γδ thymocytes developing in *in vitro*. *A,* Bar graph represent mean +/- SEM frequencies of the indicated Vδ subpopulations of total γδ T cells (left) present *in vivo* in the human thymus; *right:* mean +/- SEM frequencies of CD1a+, CD1alo and CD1a- within the Vδ1 and Vδ2 γδ T cell subsets *in vivo* in the human thymus. *B,* Kinetics flow cytometry analysis of Vδ1 and Vδ2 expression in DN2-like human thymocytes co-cultured onto the indicated Notch ligand OP9 cell lines during 31 days. Numbers in quadrants shown mean +/- SEM frequencies of the indicated cell subsets. *C,* Kinetics of absolute Vδ1+, Vδ2+ and Vδ1-Vδ2- γδ T cell numbers generated along 31 days of co-culture with the indicated Notch ligand expressing OP9 cells. Data show mean +/- SEM cell numbers +/- in 3 independent experiments.

2.8. Jag1 favors the generation of functional IFNγ-secreting γδ T cells.

Several reports in mice have pointed to Notch as a capital signal for the generation of IL-17 γδ T cells (Mukherjee et al., 2009) (Shibata et al., 2011) (Michel et al., 2012) and also for the proliferation and function of PB human IFNγ-secreting γδ T cells (Gogoi et al., 2014). Therefore, we wanted to assess the functionality of the γδ T cell subsets generated *in vitro* and

the possibility that particular Notch ligands were inducing specific γδ T cell functions. To this end, γδ T cells generated after 28 days in the different OP9-Notch ligand cultures were assayed by flow cytometry for the production of either IL-17 of IFNγ upon stimulation with anti-CD3 and IL-2 and further activation with PMA, as has been described for PB αβ and γδ T cells (Michel et al., 2012 and Fig. 41A). Given that γδ T cell generation in Dll1 and Dll4 cultures was equivalent, only Dll4 cultures were included in this study. Upon stimulation, all cultures were highly enriched in γδ T cells, of which more than 30% were fully functional, as assessed by INFγ secretion. Notably, IFNγ-producing γδ T cells were more frequent in Jag1 cultures, compared to Dll4 and Jag2 cultures (Fig. 41C). In contrast, few γδ cells produced IL-17, regardless of the Notch ligand culture (Fig. 41B, C) and generation of IL-17-secreting γδ T cells was neither observed in a IL-17 polarization assay following stimulation with plate-bound anti-CD3 Ab and a Th17-polarizing cytokine cocktail including IL-1β, TGFβ and IL-6 (Manel et al., 2008) (Keerthivasan et al., 2011) (data not shown). Given that essentially all γδ T cells derived in every culture condition have undergone maturation into CD8+ γδ T cells upon activation through the γδ TCR (Fig. 41D), **our data suggest that Jag1 is more efficient than Dll4 or Jag2 in supporting the generation of INFγ-secreting γδ T cells.** Importantly, these cells were efficiently expanded in the presence of IL-2 and, therefore, **this *in vitro* co-culture system could overcome the limitations of current protocols of generation of functional γδ T cells**. It is thus possible that using this system for large-scale generation of γδ T cells may help the development of novel and promising xeno-free approaches to ultimately generate γδ T cells suitable for clinical applications.

>> Figure 41. Notch signaling influences the generation of functionally mature IFNγ-secreting γδ T cells *in vitro*. *A.* Flow cytometry analysis of intracellular staining of IL-17 and IFNγ cytokines in either resting (*left*) or PMA-activated (*right*) human PBLs was used as positive control. Histograms show FACS analysis of human peripheral blood lymphocytes (PBLs) stimulated with anti-CD3 and IL2 for 36h and expanded in the presence of rhIL2 for 5 additional days. Activation with PMA plus ionomycin was done immediately prior cytokine staining. *B,* Flow cytometry analysis of intracellular staining of IL-17 and IFNγ cytokines in CD3- and rhIL2-stimulated human γδ thymocytes generated *in vitro* from DN2-like progenitors in the indicated Notch ligand OP9 co-cultures by day 28. Activation with PMA plus ionomycin was done immediately prior cytokine staining. Numbers in quadrants represent mean +/- SEM frequencies of the corresponding population. *C*, Bars graph diagrams representing the frequency (cell %) of INFγ+ γδ T cells and IL-17+ γδ T cells generated from DN2-like progenitors in the indicated co-cultures. Data were normalized to IFNγ+ or IL-17+ cell frequences obtained for PBL controls. **, $p < 0{,}01$. *, $p < 0{,}05$. *D*, Flow cytometry analysis of the CD8/CD4 phenotype displayed by γδ T cells analyzed in B, C and by day 28 in Notch ligand co-cultures either before (*left*) of after (*right*) anti-CD3 plus rhIL2 stimulation. Numbers in quadrants represent mean +/- SEM frequencies of the indicated cell population.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PMA-
PMA+
PBL
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<1
<1
4±1
<1
<1
3±1
<1
5±1
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49±3
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TCRγδ
IFNγ
TCRγδ
IL-17
TCRγδ
IFNγ
B
PMA-
PMA+
Total cells
Gated on TCRγδ+
Total cells
Gated on TCRγδ+
Dll4
88±3
<1
<1
88±2
<1
31±4
Jag1
92±1
<1
<1
83±5
<1
53±8
Jag2
93±3
<1
<1
89±5
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34±6
CD3
TCRγδ
TCRγδ
IL-17
TCRγδ
IFNγ
CD3
TCRγδ
TCRγδ
IL-17
TCRγδ
IFNγ
C
Relative INFγ+ γδ T cell No. (%)
1.2
1
0.8
0.6
0.4
0.2
0
**
*
Dll4
Jag1
Jag2
PBLs
Relative IL-17+ γδ T cells No. (%)
1.2
1
0.8
0.6
0.4
0.2
0
Dll4
Jag1
Jag2
PBLs
D
Day 28
CD3 + IL2
Total cells
Gated on TCRγδ+
Total cells
Gated on TCRγδ+
Dll4
31±5
5±1
62±4
4±2
30±3
92±6
94±3
<1
5±3
<1
Jag1
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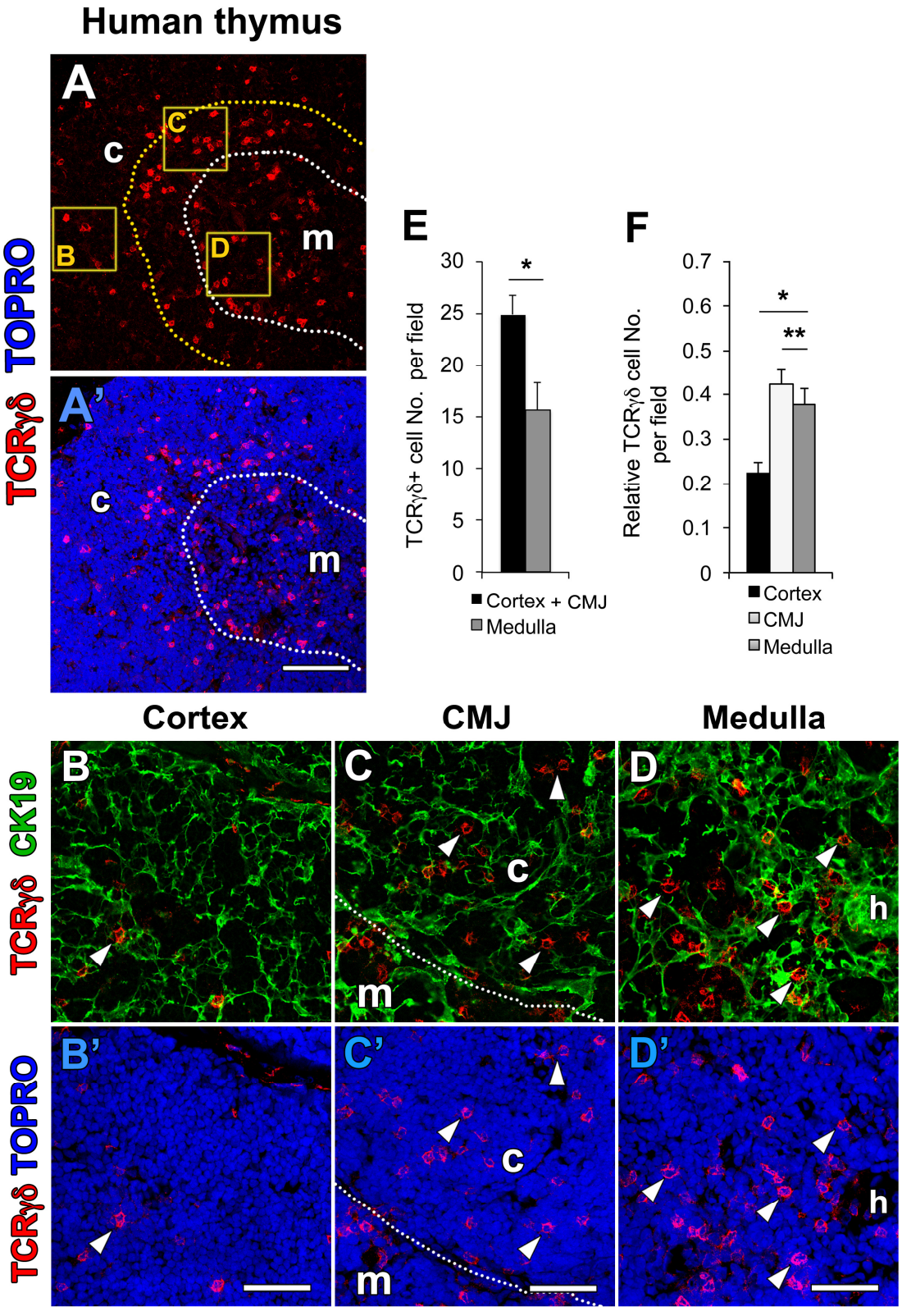
11±1
52±4
12±3
25±2
91±2
88±6
1±1
10±4
<1
Jag2
58±7
4±1
64±2
3±1
29±1
95±2
94±1
<1
5±1
<1
CD3
TCRγδ
CD8
CD4
CD3
TCRγδ
CD8
CD4

III. STUDY OF THE *IN VIVO* INTRATHYMIC DIFFERENTIATION DYNAMICS OF HUMAN γδ T CELLS.

3.1. γδ T cells preferentially accumulate at the CMJ, a Jag2-rich niche in the human thymus.

The differential function observed for Jag2 as a Notch ligand that selectively favors the generation of γδ T cells *in vitro*, as well as the expansion of their intermediate maturation stages, strongly suggests that human γδ T cells may be generated and expanded *in vivo* in Jag2-rich microenvironments. However, the particular location of γδ T cells in the human thymus and their intrathymic dynamics have been poorly addressed. Scarce reports point to the medulla as the most important niche of human intrathymic γδ T cells (Groh et al., 1989) (Falini et al., 1989). In order to confirm these data, we performed immunohistochemical analysis of postnatal human thymic tissue stained with an anti-TCRδ chain-specific monoclonal antibody (Groh et al., 1989). Anti-panCK was also used in the study to co-stain TECs. A general analysis of TCRδ expression showed a broad distribution of γδ T cells both in the cortex and in the medulla of the human thymus (Fig. 42A). A closer view to the three different functional regions of the thymic cortex: subcapsular cortex (SCC), inner cortex (IC) and corticomedulary junction (CMJ), revealed the accumulation of γδ T cells at the later. By carefully limiting this region to the narrow portion of cortical tissue close to the medulla (Fig. 42A; yellow dotted line), we could observed a precise accumulation of γδ T cells at this location, where Jag2 is highly expressed by cTECs (see Fig. 18). Conversely, detailed analyses of the remaining cortical areas, SCC and IC (both together named as "cortex"), revealed that γδ T cells are quite scarce in these cortical areas (Fig. 42B), while CMJ and thymic medulla showed notably higher numbers of γδ T cells (Fig. 42C, D). In each thymic region, panCK staining was excluded from TCRδ-positive cells, characterized as thymocytes, thus confirming antibody specificity. To formally quantify the distribution of γδ T cells in each thymic anatomic region, quantitative analyses of γδ T cell numbers were performed in no less that 10 cortical, 10 CMJ and 10 medullary images (Fig. 42B, C, D) (40x) per thymic sample ($N = 3$). Results showed that γδ T cells were significantly more abundant in the thymic cortex as a whole (SCC+ IC + CMJ regions) than in the medulla (Fig. 42E), in contrast with data presented in previous reports showing 3 times more γδ T cells located at the medulla than at the cortex (Groh et al., 1989) (Falini et al., 1989). When γδ T cell counting was done separately in the cortex (SCC + IC) and in the CMJ, we confirmed a highly significant accumulation of γδ T cells at the CMJ (Fig 42F).

Based on these data, we can conclude that human γδ T cells are more abundant at the thymic cortex but specifically in the narrow region of the CMJ, where Jag2 Notch ligand is highly expressed.



<< Figure 42. TCRγδ expression analysis in human postnatal thymus reveals accumulation of γδ T cells at the CMJ. Images show snap-frozen sections of human postnatal thymic tissue (< 17 months-old) stained for TCRγδ (red) and CK19 (green). Topro3 was used for nuclear staining (blue). *A,* General view of TCRγδ distribution in the human thymus. Thymic regions are labeled as; *c:* cortex; and *m:* medulla; *dotted line*: CMJ. Yellow squares highlight cortical, corticomedulary and medullary ROIs. Scale bar: 100μm. *B*, *C*, *D*, TCRγδ expression at the cortex, CMJ and medulla, respectively. Arrowheads indicate TCRγδ positive staining. *Dotted line:* CMJ; *h:* Hassal's corpuscle. Scale bars: 50μm. E, Bars graph shows the mean +/- SEM absolute number of TCRγδ+ cells per field (40x) at the thymic cortex (considered as cortical plus corticomedulary regions) and at the medulla. *, $p < 0,05$; **, $p < 0,01$. *F,* Bars graph shows the mean +/- SEM absolute number of TCRγδ+ on each anatomic region relative to the total number of γδ T cells per field. Data are representative of results obtained in $N \geq 10$ images (40x) per thymic region and $N \geq 3$ different tissue samples.

3.2. Different γδ T cell developmental stages are located at specific thymic niches: CMJ and medulla selectively host immature CD1a+ and mature CD1a-/lo γδ T cells, respectively.

During αβ T cell development, immature CD1a+ DP thymocytes mainly reside within the thymic cortex, but they migrate into the thymic medulla for further maturation. Whether γδ T cells follow a similar intrathymic migratory pathway is unknown. Our data have located human intrathymic γδ T cells in the CMJ and medulla. We have also demonstrated that CMJ and medulla are two very distinct niches regarding Jag1 and Jag2 Notch ligand expression. Additionally, Jag1 and Jag2 have opposing functional roles *in vitro* concerning αβ versus γδ T cell generation; indications that altogether suggest that cortical and medullary γδ T cells could be different γδ T cell populations. To test this hypothesis, we took advantage of our previous results showing a preferential generation of CD1a+ γδ T cells in response to Jag2 signaling, and ought to determine the localization in the human thymus of CD1a+ and CD1alo/- γδ T cell populations. As expected, CD1a was strongly expressed at the thymic cortex, where most immature αβ-lineage thymocytes reside. Conversely, thymic medulla showed an scarce or absent CD1a+ signal, according to the fact that it mostly hosts myeloid and mature T cells (Fig. 43A). Detailed images of CD1a and TCRγδ expression at the CMJ and medulla showed CD1a+ membrane staining in corticomedulary but not in medullary γδ T cells; only scarce medullary γδ T cells showed some low CD1a expression (Fig. 43B, C; arrowheads). This observation was confirmed by CD1a quantitative analyses (of about 50 cortical and medulary γδ T cells per tissue sample) in 10 images (63x) per postnatal thymic tissue sample ($N = 3$) (see below).

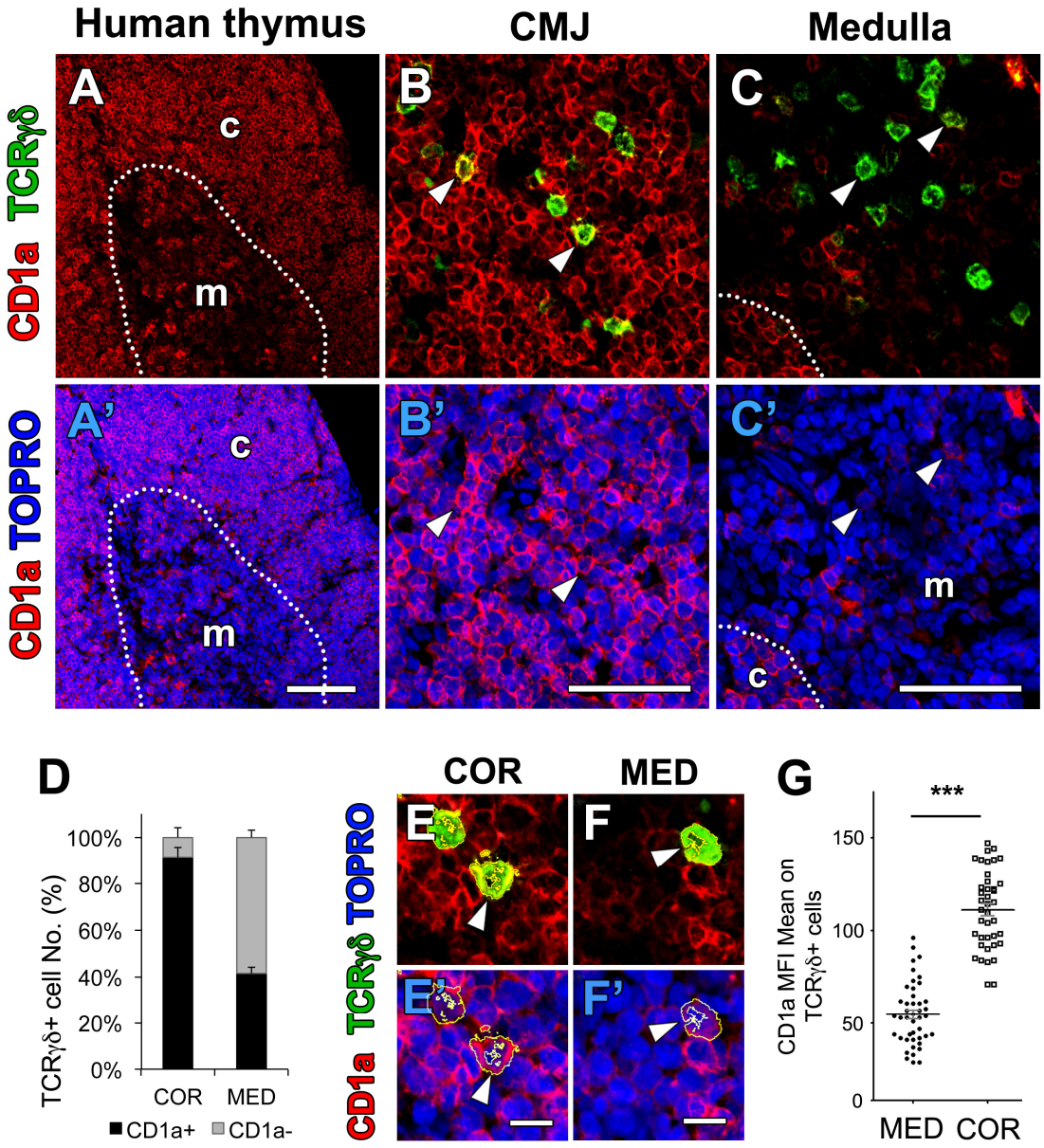


Figure 43. Different TCRγδ developmental stages defined by CD1a expression are found in different thymic niches. Images show snap-frozen sections of human postnatal thymic tissue (< 17 months-old) stained for CD1a (red) and TCRγδ (green). Topro3 was used for nuclear staining (blue). *A.* General view of CD1a distribution in the human thymus. Thymic regions are labeled as *c:* cortex; and *m:* medulla; *dotted line*: CMJ. Scale bar: 100µm. *B*, *C*, CD1a and TCRγδ expression at the CMJ and medulla, respectively. Arrowheads indicate TCRγδ+ cells co-expressing high levels of CD1a (yellow) at the CMJ, but not at the medulla. *Dotted line:* CMJ. Scale bars: 50µm. *D,* Bar graph shows mean +/- SEM frequencies of CD1a+ and CD1a- γδ T cells at the whole cortex (COR = SCC + IC + CMJ) and at the medulla (MED). Data was obtained by counting CD1a+ and CD1a- TCRγδ cells on *N* ≥ 10 images (40x) per thymic region and *N* ≥ 3 different tissue samples. *E, F,* Detail of isolated CD1a+ γδ T cells (arrowheads) present at the cortex and the medulla, respectively. Scale bars: 10µm. Yellow lines represents ROIs defined by TCRγδ expression that were used to measure CD1a MFI on cortical (*N* = 45) and medullary (N = 40) CD1a+ TCRγδ+ cells (G) on N ≥ 10 images (40x) per thymic region and *N* ≥ 3 different tissue samples. ***, p < 0,001.

Results shown in Fig. 43D, confirmed that cortical and medullary thymic microenvironments host different populations of γδ T cells, as CD1a+ immature and CD1a- mature γδ T cells are preferentially located in the CMJ and medullary thymic microenvironments, respectively. However, an important population (40%) of CD1a+ γδ T cells was also located at the thymic medulla. To assess whether these medullary and cortical CD1a+ γδ T cells were equivalent cell subsets, we performed quantitative analyses aimed at defining CD1a expression levels (MFI) in CD1a-expressing cortical and medullary γδ thymocytes. No less than $N = 45$ γδ T cells per postnatal thymic tissue sample ($N = 3$) were analyzed as following. As shown in Fig. 43E, F, ROIs (yellow line) were defined by TCRγδ intensity threshold (Otsu, 1979) on individual CD1a+ γδ T cells. Results shown in Fig. 43G demonstrated that CD1a expression levels of medullary CD1a+ γδ T cells were significantly lower than their cortical counterparts, suggesting that they represent distinct populations of γδ T cells. Based on these results, we can propose that medullary CD1a+ γδ T cells are an intermediate developmental stage between cortical CD1a+ and medullary CD1a- γδ T cells. **Altogether, our data show that two different thymic niches, the CMJ and the medulla, host γδ thymocytes at successive developmental stages, suggesting that CD1a+ γδ thymocytes migrate into the thymic medulla while downregulating CD1a expression, thus following a dynamic maturation pathway similar to that of αβ T cells.**

3.3. Vδ1 and Vδ2 γδ T cells are located at different thymic microenvironments.

Our results above (Fig. 40A) indicate that Vδ1+ thymocytes are mostly CD1a+ cells, which represent more than 50% of total γδ T cells in the human thymus. Conversely, Vδ2+ cells are quite scarce in the human thymus and most of them display a mature CD1alo/- γδ T cell phenotype. Therefore, we next investigated whether the differential distribution of CD1a+ and CD1a- γδ thymocytes in the human thymus, can be extended to Vδ1+ and Vδ2+ intrathymic populations. As expected, Vδ1 cells were shown mostly located in cortical areas, although the medulla also hosts some Vδ1 cells (Fig. 44A). A detailed analysis of the three thymic niches (Cortex, CMJ and Medulla) showed few scattered Vδ1 cells at the cortex and medulla, but numerous Vδ1 cells at the CMJ (Fig. 44B, C, D). Quantitative analyses of no less than 10 images (63x) per postnatal thymic tissue sample ($N = 3$), confirmed that Vδ1 cells are mostly located at cortical regions (cortex + CMJ regions), and accumulate at the CMJ (Fig. 44E). Equivalent analyses of Vδ2+ cell distribution showed that the human thymus host very few Vδ2 cells (Fig. 45A), and they are mostly confined to the medullary microenvironment (Fig. 45D, E). **Altogether this data demonstrate that Vδ1 and Vδ2 γδ T cells, which are mostly CD1a+ and CD1alo/-, respectively are hosted in two different thymic niches, either the cortex or the medulla.**

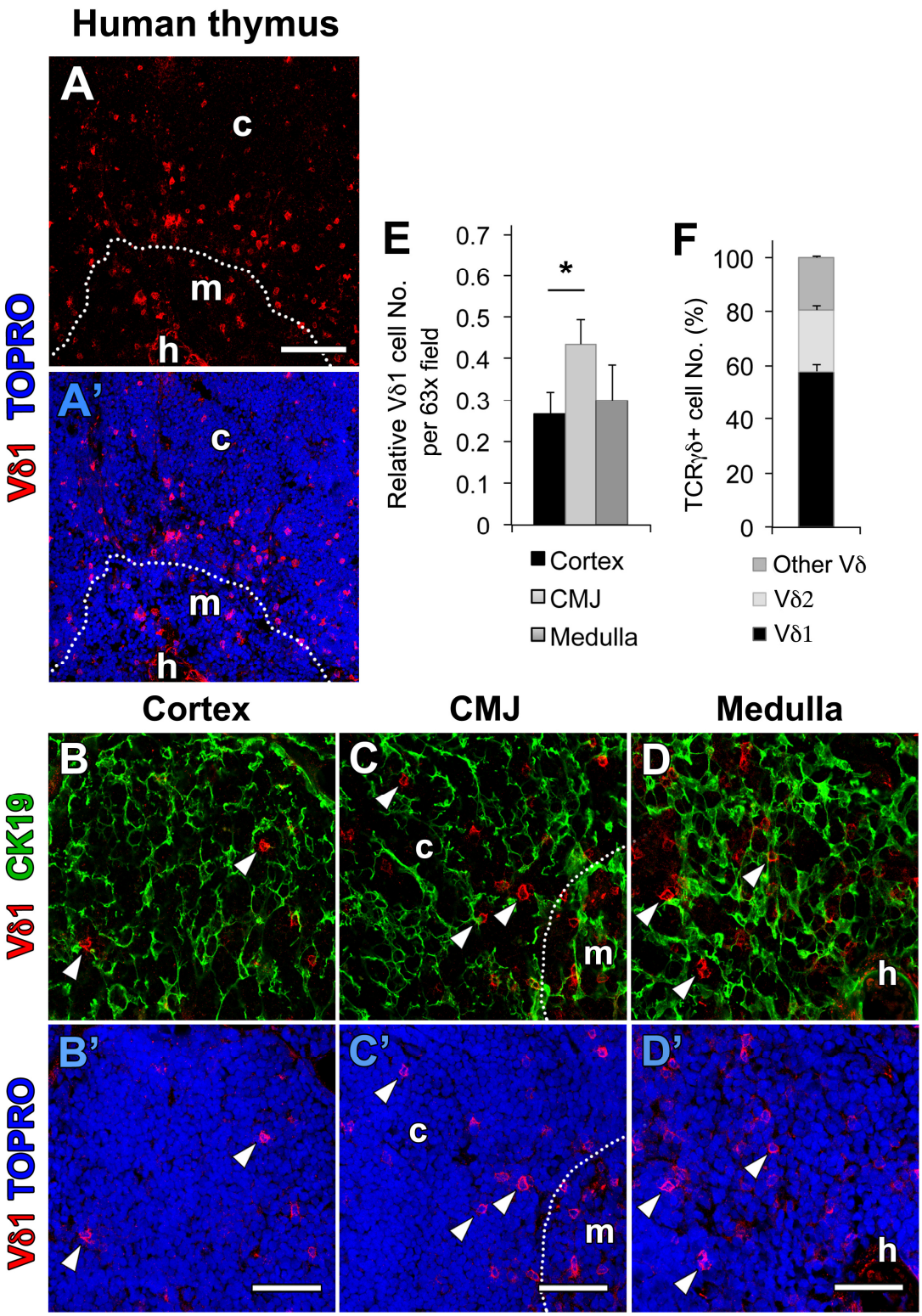


Figure 44. Vδ1 expression in human postnatal thymus. Images show snap-frozen sections of human postnatal thymic tissue (< 17 months-old) stained for Vδ1 (red) and CK19 (green). Topro3 was used for nuclear staining (blue). *A,* General view of Vδ1 expression in the human thymus. Thymic regions are labeled as; *c:* cortex; and *m:* medulla; *dotted line*: CMJ. Scale bar: 100µm. *B, C, D*, Vδ1 expression at the cortex, CMJ and medulla, respectively. Arrowheads indicate Vδ1 positive staining. *Dotted line:* CMJ; *h:* Hassal's corpuscle. Scale bars: 50µm. *E,* Bars graph showing the mean +/- SEM absolute number of TCRγδ+ on each anatomic region relative to the total number of γδ T cells per field. *F,* Bar graph shows the mean +/- SEM frequencies of the different Vδ subpopulations present *in vivo* in the human thymus. Data are representative of results obtained from $N \geq 10$ images (40x) per thymic region and $N \geq 3$ different tissue samples.

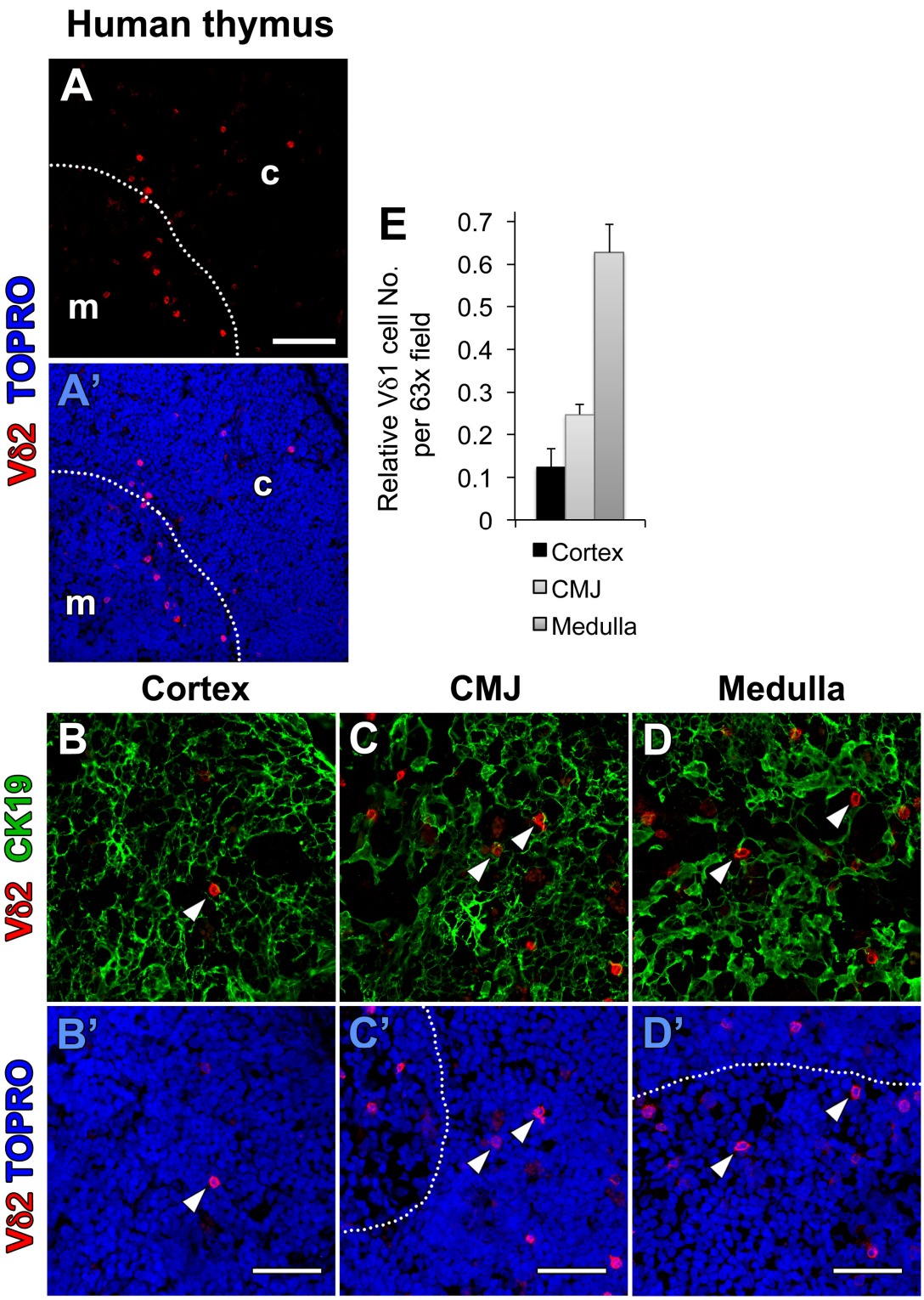


Figure 45. Vδ2 expression in human postnatal thymus. Images show snap-frozen sections of human postnatal thymus (< 17 months-old) stained for Vδ2 (red) and CK19 (green). Topro3 was used for nuclear staining (blue). *A,* General view of Vδ2 expression in the human thymus. Thymic regions are labeled as; *c:* cortex; and *m:* medulla; *dotted line*: CMJ. Scale bar: 100μm. *B*, C, *D*, Detailed Vδ2 expression at the cortex, CMJ and medulla, respectively. Arrowheads indicate Vδ2 positive staining, excluded from panCK+ staining. *Dotted line:* CMJ; *h:* Hassal's corpuscle. Scale bars: 50μm. *E*, Bars graph shows the mean +/- SEM absolute number of γδ T cells on each anatomic region relative to the total number of γδ T cells per field. Data were obtained from N ≥ 10 images (40x) per thymic region and *N* ≥ 3 different tissue sections.

3.4. Intrathymic γδ T cells express Notch1 and Notch3 receptors.

We have previously shown, that Notch receptors are distributed *in vivo* in different thymic niches and expressed by different cell types. Notch1 is mainly expressed by TECs and scattered cortical thymocytes, while Notch3 is exclusively expressed by thymocytes at the cortex, and by TECs at the medulla. At the cortex, Notch1 and Notch3 receptors may interact mostly with the Jag2 Notch ligand expressed on cTECs, which specifically enhance γδ T cell generation and impairs αβ development. Recently, it has been shown than differential Notch receptor-ligand interactions mediate αβ versus γδ T cell development by inducing differential Notch signaling strength and that, particularly Jag2, induces the strongest Notch signal through the interaction with the Notch3 receptor (Van de Walle et al., 2013). Despite these findings, Notch receptors expression on human intrathymic γδ T cells has not been yet addressed. The *in vivo* localization of immature (CD1a+ DP and/or CD1a+ CD4+) gd T cells at the CMJ, where Jag2 and Notch3 expression are maximal, aimed us to investigate Notch1 and Notch3 receptors expression *in vivo* by different γδ T cells subtypes.

Phenotypic analyses of Notch1 and Notch3 expression on human thymocytes showed a differential distribution of these receptors on intrathymic γδ T cells, as Notch1 was expressed in about 70% of γδ T cells, while low levels of Notch3 were detected in up to 35% of γδ thymocytes (Fig. 46A). Multiparameter flow cytometry analyses of the distribution of both receptors on distinct γδ T cell subtypes revealed that, as shown for αβ T-lineage cells (Fig. 46B), Notch1 and Notch3 were expressed by all γδ thymocyte subsets, although at different frequencies (Fig. 46C). Notch1 was significantly underrepresented in DN γδ T cells, compared with DP γδ thymocytes, which showed the higher frequency of Notch1+ cells, but it was also expressed in a considerable proportion of CD4+ and CD8+ SP γδ thymocytes. More importantly, Notch3 expression was significantly enriched in DP γδ thymocytes compared to all the other γδ T cell subsets (Fig. 46C). **Altogether, these findings are compatible with an specific role for Notch3 at the immature DP stage of γδ T cell development.**

We also found that Notch3 expression, poorly addressed in αβ-lineage thymocytes (Felli et al., 1999), was enriched as well in DP αβ-lineage cells. Nevertheless, a similar proportion of Notch3+ cells were detected in the upstream thymocyte subset of DN precursors that still lack a particular TCR (Fig. 46B). Therefore, Notch3 seems to be selectively downregulated during positive selection of SP αβ thymocytes; whereas, as previously shown, Notch1 is early downregulated during transition to the DP αβ thymocyte stage (Huang et al., 2003) (Fiorini et

al., 2009) (Gonzalez-Garcia et al., 2009). **Collectively, these findings concur with our observation that Notch3 is strongly expressed *in vivo* in the hematopoietic population of mostly DP thymocytes located at the cortical thymic niche (see Fig. 28D, E), in contrast with Notch1 which is early downregulated prior the DP stage.**

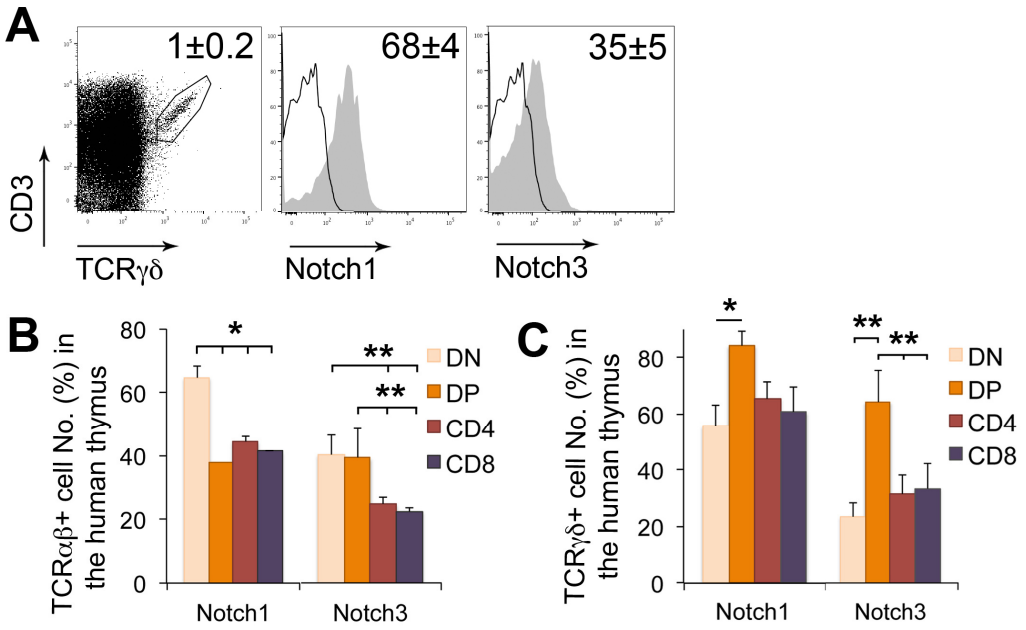


Figure 46. Notch1 and Notch3 receptor expression in human intrathymic γδ T cells populations. *A.* Flow cytometry analysis of Notch1 and Notch3 receptor expression in electronically gated human intrathymic γδ T cells. Numbers in histograms show the mean +/- SEM frequencies of γδ thymocytes expressing Notch1- or Notch3. *B,* Bars graph shows the mean +/- SEM frequencies of Notch1 and Notch3 expression on CD4/CD8-defined γδ thymocyte subsets (DN, CD4+, DP and CD8+). Data are representative of 2 - 4 independent experiments. **, $p < 0,01$; *, $p < 0,05$.

3.5. Intrathymic γδ T cell subsets differentially express the CXCR4 chemokine receptor.

The differential distribution of immature and mature γδ T cells within the human thymus observed in this study suggests that the developmental maturation of γδ thymocytes may be accompanied by a selective migration of immature CD1a+ γδ thymocytes from the CMJ to the medulla. Therefore, it was expected that, as reported for thymocytes developing along the αβ T cell lineage (Bleul and Boehm, 2000) (Norment and Bevan, 2000) (Annunziato et al., 2001) (Liu et al., 2006) (Petrie and Zuniga-Pflucker, 2007) expression of chemokine receptors could play a crucial role in that process. However, the relationship of γδ T cell differentiation and their intrathymic dynamics is less well understood.

Regarding chemokine/chemokine receptor pathways involved in thymocyte maturation, it is known that CXCL12 (SDF1) is abundantly expressed at the CMJ, where it is involved in thymus seeding by BM-derived progenitors (Aiuti et al., 1999) (Plotkin et al., 2003). Cortical transmigration is regulated by CCR9-CCL25 (Campbell et al., 1999) (Norment et al., 2000), while CCR4 and CCR7, and their respective ligands CCL22 and CCL19/CCL21, are abundant at the thymic medulla where they orchestrate cortico-medullary migration of post-selected DP and CD4+ and CD8+ SP αβ thymocytes (Chantry et al., 1999) (Witt and Robbins, 2005). Regarding γδ T cells, studies with CCR7- and CCR9-deficient mice showed that CCR7 and CCR9 expression was important for γδ T-cell localization within the thymic medulla or cortex, respectively (Reinhardt et al., 2014). Of them, CCR9 expression is notably expressed in murine γδ thymocytes (Uehara et al., 2002). Based on these grounds, the expression of CCR9 and CXCR4 was studied by FACS in *ex-vivo*-isolated human γδ thymocytes, with the aim of investigating whether discrete human γδ developmental stages have a differential expression of chemokine receptors. In contrast to murine data (Uehara et al., 2002), no CCR9 expression was detected in human thymocytes, including γδ thymocytes (Fig. 47A, B, *left*). Conversely, CXCR4 was expressed in a considerable proportion (up to 40%) of total human thymocytes, and particularly in DN and DP thymocytes (Fig. 47B, C, right), as reported before in mice (Plotkin et al., 2003). Detailed analyses of CXCR4 expression in γδ thymocyte subsets, revealed a preferential expression of this chemokine receptor in the DP and CD4 γδ cell subsets with an immature CD1a+ phenotype (Fig. 47E,), **thus suggesting that CXCR4 downregulation parallels cortico-medullary migration and γδ thymocyte maturation.**

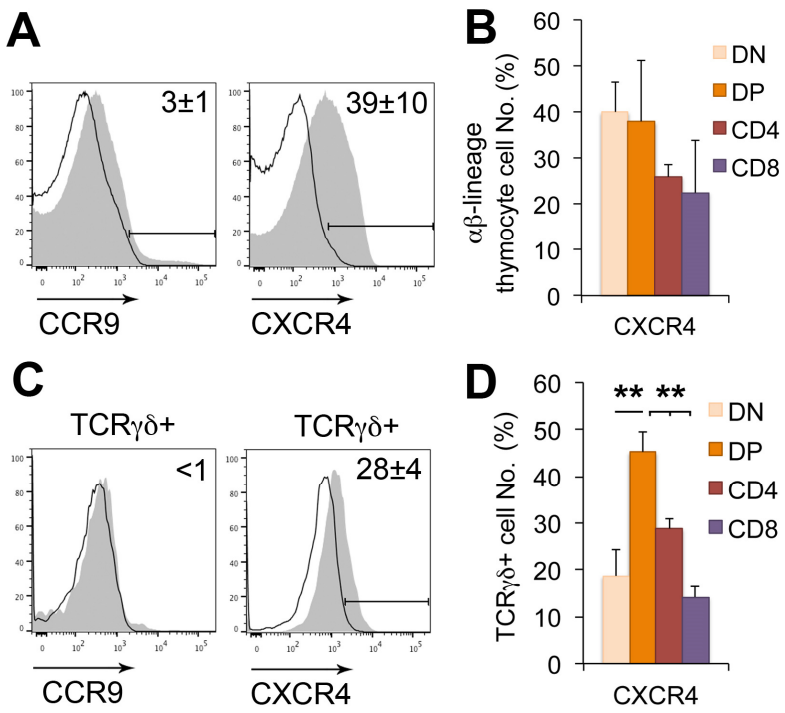


Figure 47. CCR9 and CXCR4 chemokine receptor expression in human intrathymic γδ T cell populations. *A,* Flow cytometry analysis of CCR9 and CXCR4 expression on human αβ-lineage thymocytes. Numbers in histograms indicate mean +/- SEM frecuencies of cells expressing CCR9 or CXCR4. *B,* Bar graph shows the mean +/- SEM frequencies of CXCR4 expression on CD4/CD8-defined γδ thymocyte subsets (DN, CD4+, DP and CD8+). *C,* Flow cytometry analysis of CCR9 and CXCR4 expression on human intrathymic γδ T cells. Numbers in histograms indicate mean +/- SEM frecuencies of cells expressing CCR9 or CXCR4. *D,* Bar graph shows the mean +/- SEM frequencies of CXCR4 expression on CD4/CD8-defined γδ thymocyte subsets (DN, CD4+, DP and CD8+). Data shown are representative of 2 - 4 independent experiments. **, $p < 0,01$.

IV. ROLE OF NOTCH IN THYMIC EPITHELIAL CELL BIOLOGY.

Crosstalk between developing thymocytes and TECs is key to achieve full maturation of both cell types (Fig. 5). Several conserved signalling pathways play a capital role in TEC development and maturation (see Introduction 3.4), and our studies showing *in vivo* expression of Notch receptors on TECs (see Results 1.4) suggest, although it remains to be formally established, that Notch signalling may be one of those crucial pathways for TEC development. In order to study the role of Notch signalling in TEC biology, we have undertaken two different approaches. First, we performed immunofluorescence studies to analyse the spatio-temporal pattern of Notch activation within the thymus in both mouse and human TECs. Then, to further dissect Notch function in TEC development and homeostasis. We moved to an *in vivo* system and used a conditional murine model in which Notch signalling was specifically abrogated in TECs.

4.1. The Notch signaling pathway is active in TECs.

In order to assess whether Notch signalling is active *in vivo* in human postnatal TECs, we analysed the expression of the well-established Notch activation target Hes1 in human postnatal thymus tissue sections. This approach revealed a broad and quite homogeneous pattern of Hes1 nuclear expression throughout the thymus, although the thymic medulla exhibited higher frequency of Hes1+ cells than the cortex (Fig. 48A). Detailed analyses of singular thymic regions, including the subcapsular cortex (SCC), inner cortex (IC) and medulla (MED), with the aid of the pancytokeratin (panCK) epithelial cell marker, allowed us to identify both Hes1+ cTECs and Hes1+ mTECs in the human thymus (Fig. 48B, C, D); expression pattern that was conserved in mice (Fig. 51B, C). Hes1+ cTECs with a strong nuclear staining (arrowheads) were found throughout the SCC and IC, and scarce non-epithelial Hes1+ cells, likely developing thymocytes, were also found at these locations (arrows) (Fig. 48B, C). Hes1+ TECs were quite frequent at the thymic medulla and accumulated in Hassal's corpuscles (HCs), where mTECs displayed the highest Hes1 expression levels (Fig. 48D). Similar results were obtained in murine postnatal thymus (Fig. 51B, C). Collectively, our **immunohistochemistry analyses of intrathymic Hes1, expression provide the very first evidence of Notch signaling activation in human and murine TECs located at the cortical and medullary microenvironments.**

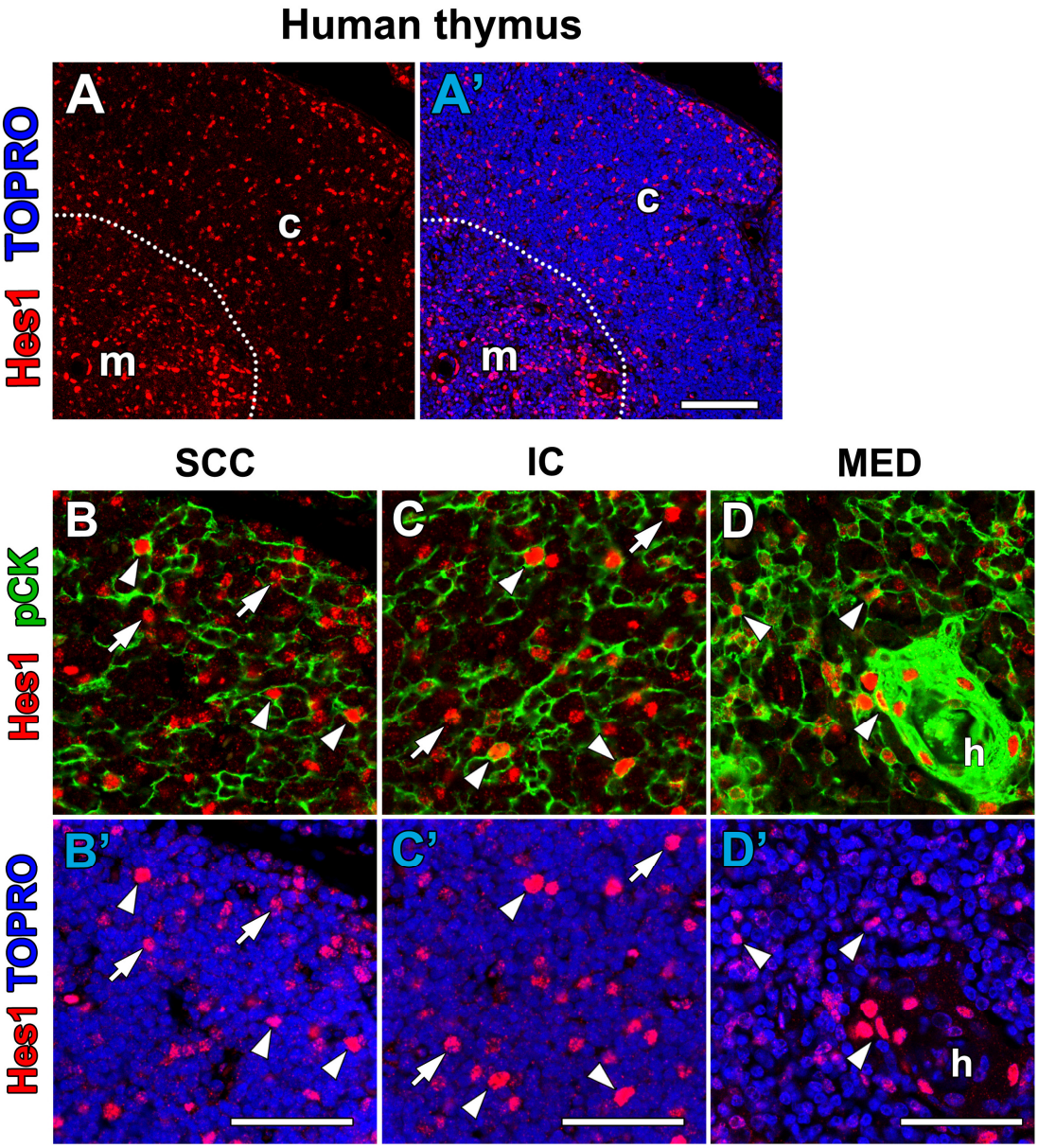


Figure 48. Notch signaling pathway is active in cortical and medullary TECs. Images show FFPE sections of human postnatal thymic tissue (< 17 years-old) stained for the Notch target Hes1 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A,* General view of Hes1 expression in the human thymus. Thymic regions are labeled as *c:* cortex; and *m:* medulla. *Dotted line:* CMJ. Scale bar: 100µm. *B, C*, *D*, Hes1 expression at the subcapsular cortex (SCC), the inner cortex (IC) and the medulla (MED), respectively. Arrowheads indicate Hes1 expression in TECs (panCK+). Arrows indicate Hes1 expression in non-epithelial (panCK-) cells. *h:* Hassal's corspuscles. Scale bars: 50µm. Images shown are representative of results obtained from $N \geq 3$ tissue samples.

4.2. Notch1 activation in mTECs increases with age and correlates with thymic involution in both humans and mice.

In order to formally prove that Notch1 signaling is actually activated in Notch1-expressing cTECs and mTECs (Fig. 28B, C), we directly analyzed the nuclear expression of the active form of Notch1 receptor in TECs labeled with a panCK antibody. Conversely to that found at the cortex (Fig. 29B, C, D), where ICN1+ cells were exclusively thymocytes and rare subcapsular cTECs, ICN1+ cells in the medulla were exclusively mTECs (Fig. 49A). As expected from our Hes1 expression results, mTECs surrounding or forming HCs were ICN1+. While the role of HCs is still a matter of debate, the observation that their numbers and size increase with age, has suggested that they are related with thymic involution. In this context, our findings might support a role for Notch signaling in HC formation as well as in mTEC terminal differentiation and senescence. In order to test this hypothesis, we next analyzed ICN1 expression in human involuted thymic tissue in comparison with normal postnatal thymic tissue. As shown in Fig. 49B, detailed examination of ICN1-labeled samples revealed a frequency of ICN1+ mTECs significantly higher in involuted than in postnatal human thymus. Equivalent results were obtained in the murine thymus (not shown). Quantitative analyses of ICN1+ mTEC numbers in postnatal (N = 3) and involuted (N = 2) thymic samples ($N \geq$ 15 images per sample; 63x) (Fig. 49C), confirmed this observation and revealed that more than 60% of total mTECs in the involuted thymus display an active Notch1 signaling pathway.

The differences in ICN1+ observed in young and aged human thymus samples, prompted us to investigate the kinetics of Notch signaling activation during postnatal life. To this end, we analyzed ICN1 expression in mTECs from 0.5, 1.5, 3, 5 and 9 months-old murine thymic tissue (N = 3 tissue samples per age). Analysis of at least 15 images per sample (63x) showed low frequencies of mTECs expressing active Notch1 in young mice (0.5 and 1.5 months-old). By 3 months of age, numbers of mTECs expressing ICN1 increase significantly, and by 5 and 9 months-old they increase further. **Therefore, our results are compatible with a role for Notch1 in mTEC homeostasis and/or age-related involution.** Whether Notch receptors other than Notch1 also contribute to Notch signaling in TECs deserves further studies using suitable antibodies currently not available.

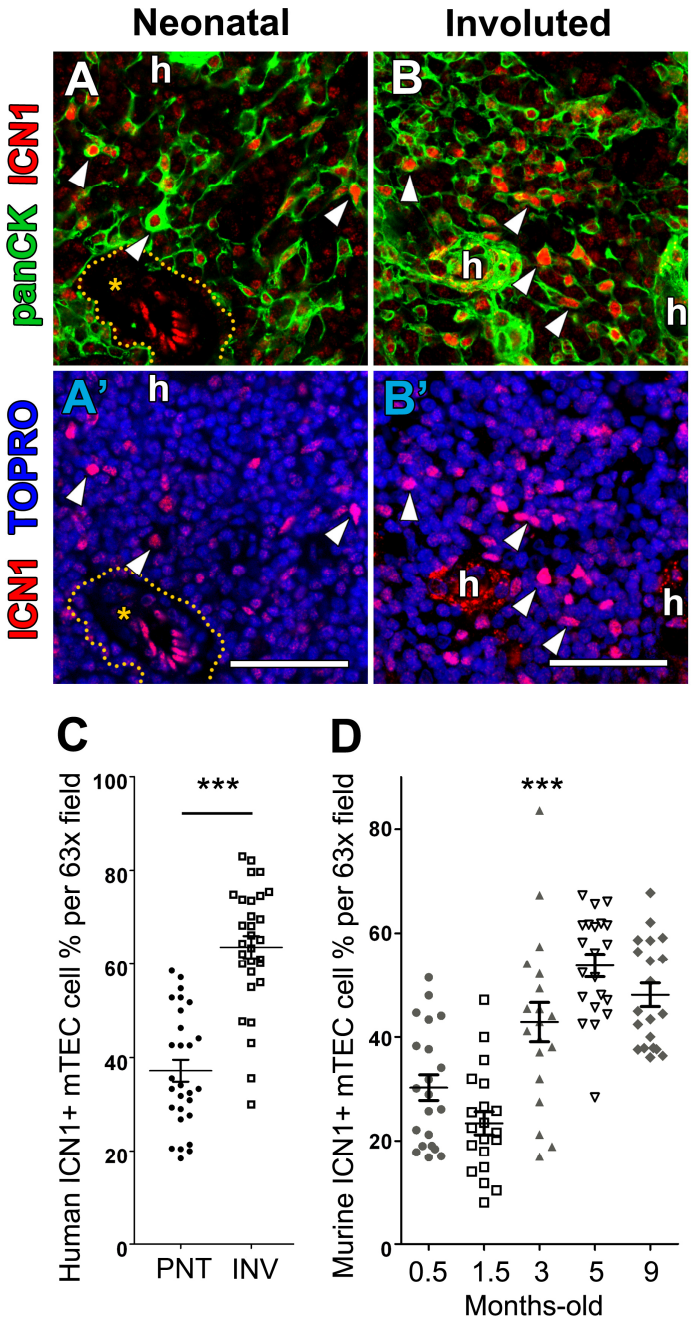


Figure 49. Notch1 activation at the medulla increases with thymic involution. Images show FFPE sections of human postnatal (< 17 years-old) and human involuted (> 6 years-old) thymic tissue stained for the intracellular active form of Notch1 (ICN1) (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A, B,* ICN1 expression at the medulla, respectively, of the postnatal and involuted human thymus. *Dotted line:* PVS; *asterisk:* ICN1+ thymic endothelium. Scale bar: 50µm. *C,* Graph shows mean +/- SEM frequencies of ICN1+ mTECs in human postnatal and involuted thymic medulla. ***; p < 0,0001. *D,* Graph shows mean +/- SEM frequencies of ICN1+ mTECs in the murine thymus along time, ranging from postnatal (2 wks-old) to involuted (9 months-old) thymi. ***; p < 0,0001. Data were obtained by counting total mTECs and ICN1+ mTECs in *N* ≥ 10 images (25x) per tissue sample (*N* = 3).

4.3. Conditional inactivation of Notch signaling in epithelial cells.

To better understand the implications of Notch signaling in TEC biology, we developed a conditional mouse model of RBPjk deletion in epithelial cells (designated here as RBPjk-KO^{TEC} mice) by crossing RBPjk$^{Lox/Lox}$ mice with FoxN1-Cre mice (Fig. 50A). In this mice, RBPjk deletion is specifically driven by the FoxN1 promoter which allow expression of the Cre recombinase in the thymus when FoxN1 is expressed at Ed11,5 of thymic development. In order to confirm that Cre recombinase was expressed by TECs, FoxN1-Cre mice were crossed with mice carrying the LoxP site-flanqued LacZ allele in homozygosis under the constitutive and ubiquitously expressed Rosa26 promoter (Rosa26$^{LoxLacZ+/+}$). β-galactosidase expression was assessed in histologic sections of the resulting Cre-LacZ reporter mice thymi. Positive β-galactosidase staining was observed at discrete patches in the thymic cortex (Fig. 50B) and the thymic medulla (Fig. 50B), **thus confirming Cre recombinase expression in cortical and medullary TECs.**

As detailed at the Introduction, FoxN1 is expressed in epithelial tissues other than the thymus, as gut and skin. Accordingly, RBPjκ-KO^{TEC} mice developed macroscopic skin lesions and hair defects with age, which were evident around the 8th month of life (Fig. 50D). Aged RBPjk-KO^{TEC} mice showed clear signs of disease. Although their average weight was not significantly different from that of WT mice (data not shown), they appeared thinner and smaller due to a lack of proper hair density. Detailed examination of the RBPjk-KO mice revealed rounded lesions at face, ventral, footpad and tail skin (Fig. 50E, F, G and H, respectively). Microscopic examination of these lesions showed a clear disorganization of the skin that was already appreciable at younger ages and showed signs of inflammation, leukocyte infiltration, hair follicle hyperproliferation and keratin cysts (Fig. 50I) coincident with skin defects shown before in epithelial Notch-deficient mice (Demehri et al., 2009). **Therefore, this skin phenotype provides formal proof of Notch deletion in the epithelium in our conditional KO model.**

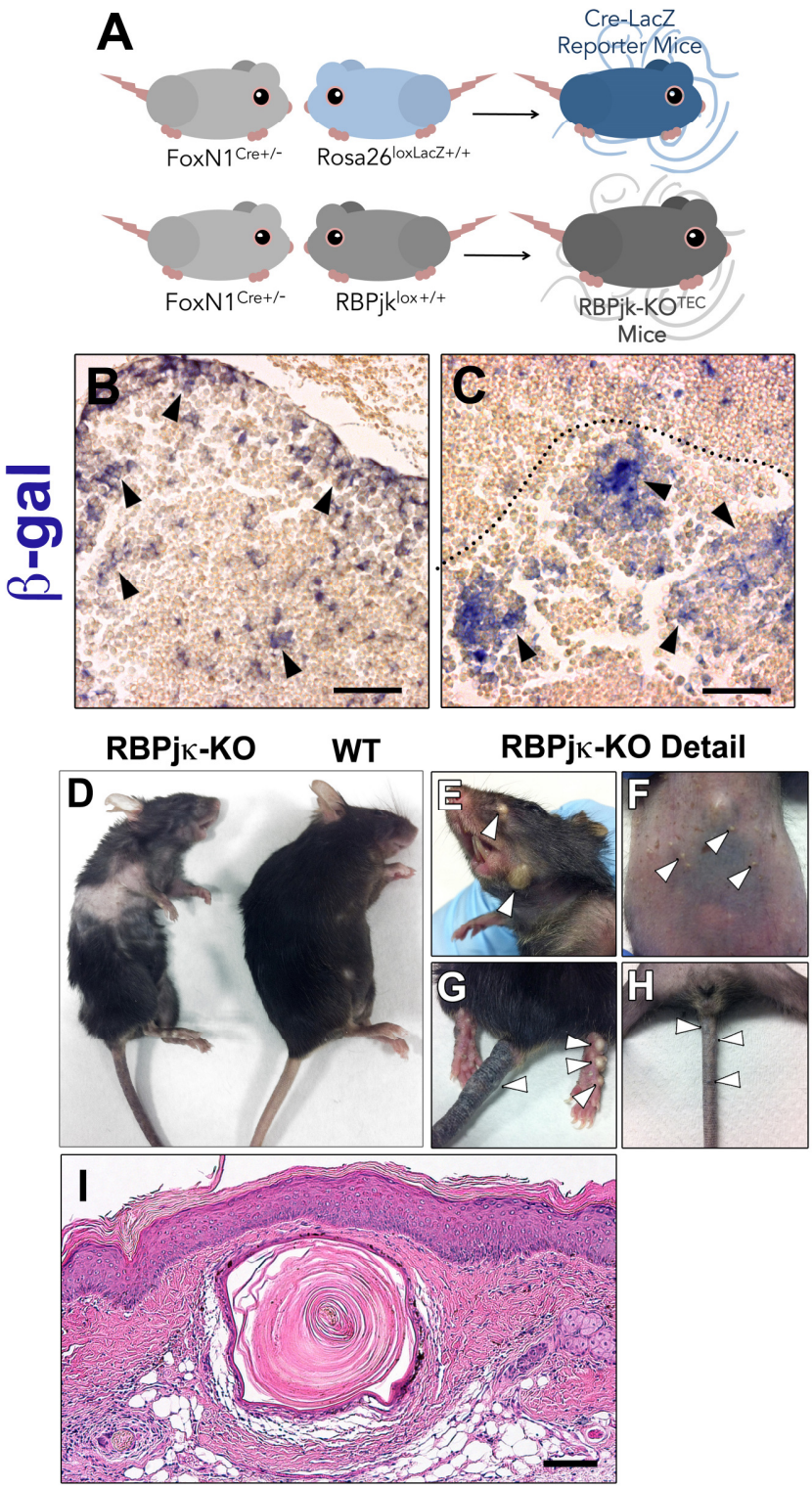


Figure 50. Murine model for the study of Notch signaling on TECs. A, *Up;* Cre-LacZ reporter mice that express β-galactosidase under the epithelial transcription factor promoter FoxN1 which result from crossing FoxN1-Cre$^{+/-}$ with Rosa26$^{LoxLacZ+/+}$ mice. *Down;* RBPjκ conditional KO mice resulting from crossing FoxN1-Cre$^{+/-}$ with RBPjkLox+/+ mice. In these mice RBPjκ Notch signaling repressor is specifically deleted from Foxn1-expressing epithelial cells. In the thymus, RBPjκ is deleted from Ed11,5, when Foxn1 is expressed in TECs. *B*, *C*, Images show cortical and medullary details, respectively, of β-galactosidase staining of 3 days-old Foxn1-Cre-LacZ thymus. Arrowheads show Cre-recombinase expressing TECs and thus β-galactosidase positive staining (blue). Dotted line: CMJ. Scale bar: 200μm. *D,* General view of aged (9 months-old) WT and RBPjκ-KO^{TEC} mice. *E, F, G, H,* Images show skin lesions (arrowheads) consequence of Notch signaling abrogation in the skin epithelium. *I,* Hematoxylin/eosin staining of tissue slides from footpad skin showing a keratin cysts.

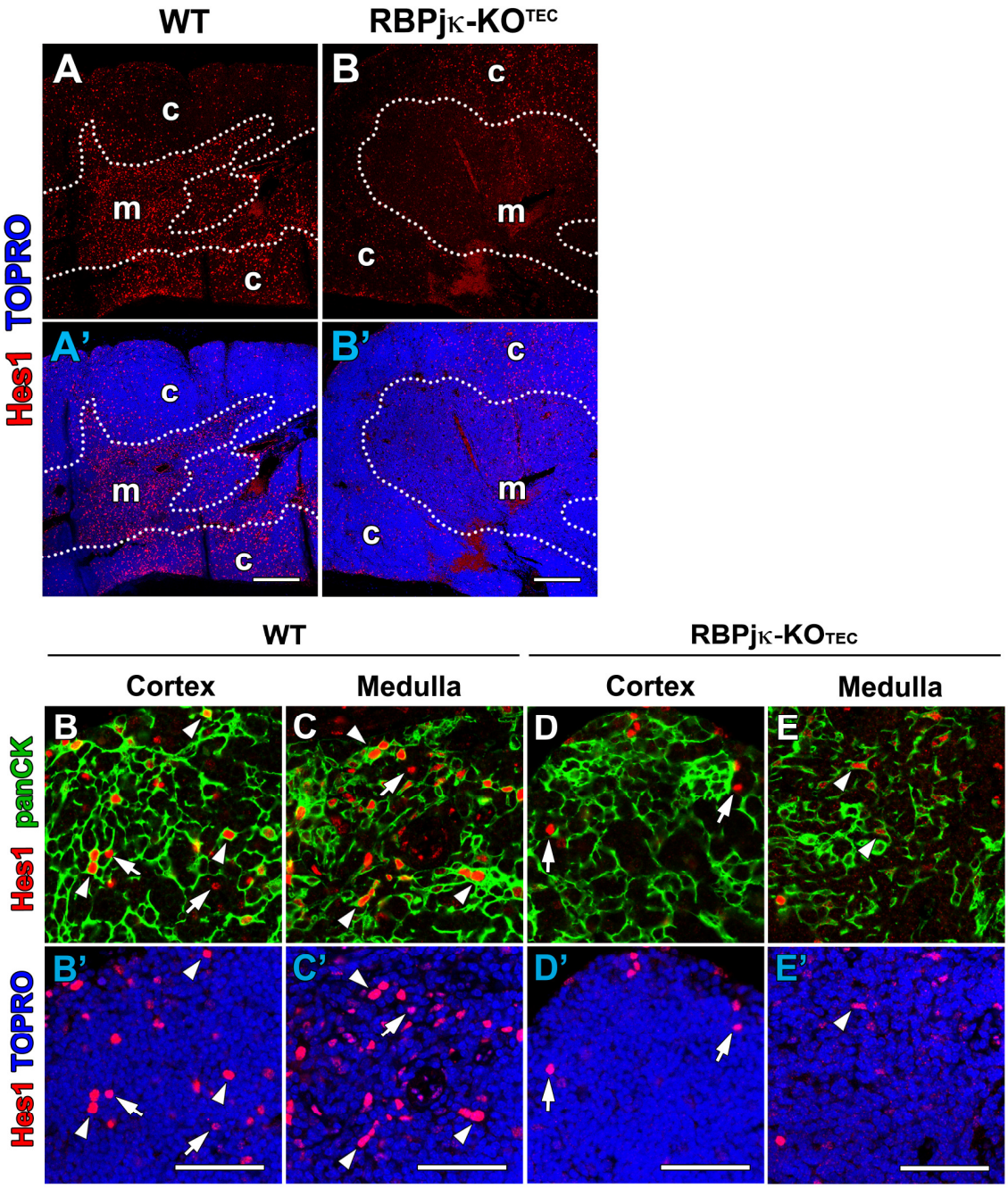


Figure 51. Notch signaling is specifically abrogated in RBPjκ-KO TECs. Images show FFPE sections of murine postnatal thymic tissue (5 months-old) stained for the Notch target Hes1 (red) and panCK (green). Topro3 was used for nuclear staining (blue). *A*, *B*, General view of Hes1 expression in WT and RBPjκ-KO^{TEC} thymi. Thymic regions are labeled as *c:* cortex; and *m:* medulla. *Dotted line:* CMJ. Scale bar: 200μm. *B*, *C*, Expression of Hes1 and panCK in WT mice at the thymic cortex and the medulla, respectively. *D*, *E*, Expression of Hes1 and panCK in RBPjκ-KO^{TEC} mice at the thymic cortex and medulla, respectively. Arrowheads indicate nuclear Hes1-positive staining in cTECs and mTECs. Arrows indicate Hes1 positive staining in non-epithelial cells (thymocytes). Scale bars: 50μm.

4.4. Activation of Notch signaling is impaired in TECs from RBPjκ-KO^{TEC} mice

In order to provide formal proof of RBPjκ deletion in TECs from RBPjκ-KO^{TEC} mice, we performed comparative immnunohistological analyses of Hes1 expression in thymic tissue samples from RBPjκ-KO^{TEC} and WT mice (Fig. 51). Mice at different ages ranging from 3 to 11 months were analyzed (N = 8) and consistent results were obtained in terms of Hes1 expression regardless of their age. The analysis of 5 month-old RBPjκ-KO^{TEC} thymus samples (Fig. 51), revealed a drastic decrease of Hes1 staining at the cortex, and especially at the medulla, where Hes1+ TECs accumulate in WT animals (Fig. 51A). Detailed examination of the cortical niche (Fig. 51B, D), confirmed the abrogation of Hes1 expression in panCK+ cTECs; while the minor proportion of Hes1+ cells found in the thymic cortex were panCK- thymocytes (Fig. 51D), thus confirming the specific deletion of RBPjκ in TECs. Similarly, a strong reduction of Hes1+ mTEC numbers was observed in the RBPjκ-KO^{TEC} thymic medulla (Fig. 51C, E), although some Hes1+ mTECs that display lower expression levels and different morphology than WT mTECs were detected as well (arrowheads). **Overall, these data confirm that (1) Hes1 is a good marker for studying Notch pathway activation in the thymus, and (2) that our model of conditional deletion of RBPjκ in TECs is efficient and specific.**

4.5. Abrogation of Notch signaling in TECs disrupts the medullary thymic microenvironment.

Macroscopic examination of RBPjκ-KO thymi revealed no significant differences although they were slightly smaller than WT thyme. Several reports have shown that mTECs derive from FoxN1+ cTEC and mTEC bipotent progenitors that express cTEC markers (Bleul et al., 2006) (Anderson et al., 2009) (Alves et al., 2014). Thymic medulla develops clonally from cTEC-derived mTECs islets (Rodewald et al., 2001), that enlarge and fuse at some point of development forming an histologically continuous niche. The molecular milieu of signals that regulate medullary formation and thus cortico-medulary compartimentalization is still partially unveiled (see Introduction 1.2); but our results suggest that Notch signaling could have an important role in mTEC biology, at least during postnatal life. To investigate this possibility, we performed histomorphometric studies of the thymus aimed to establish a detailed comparison of the cortical and medullary areas (*ca* and *ma*) of thymus samples from WT and RBPjκ-KO^{TEC} mice.

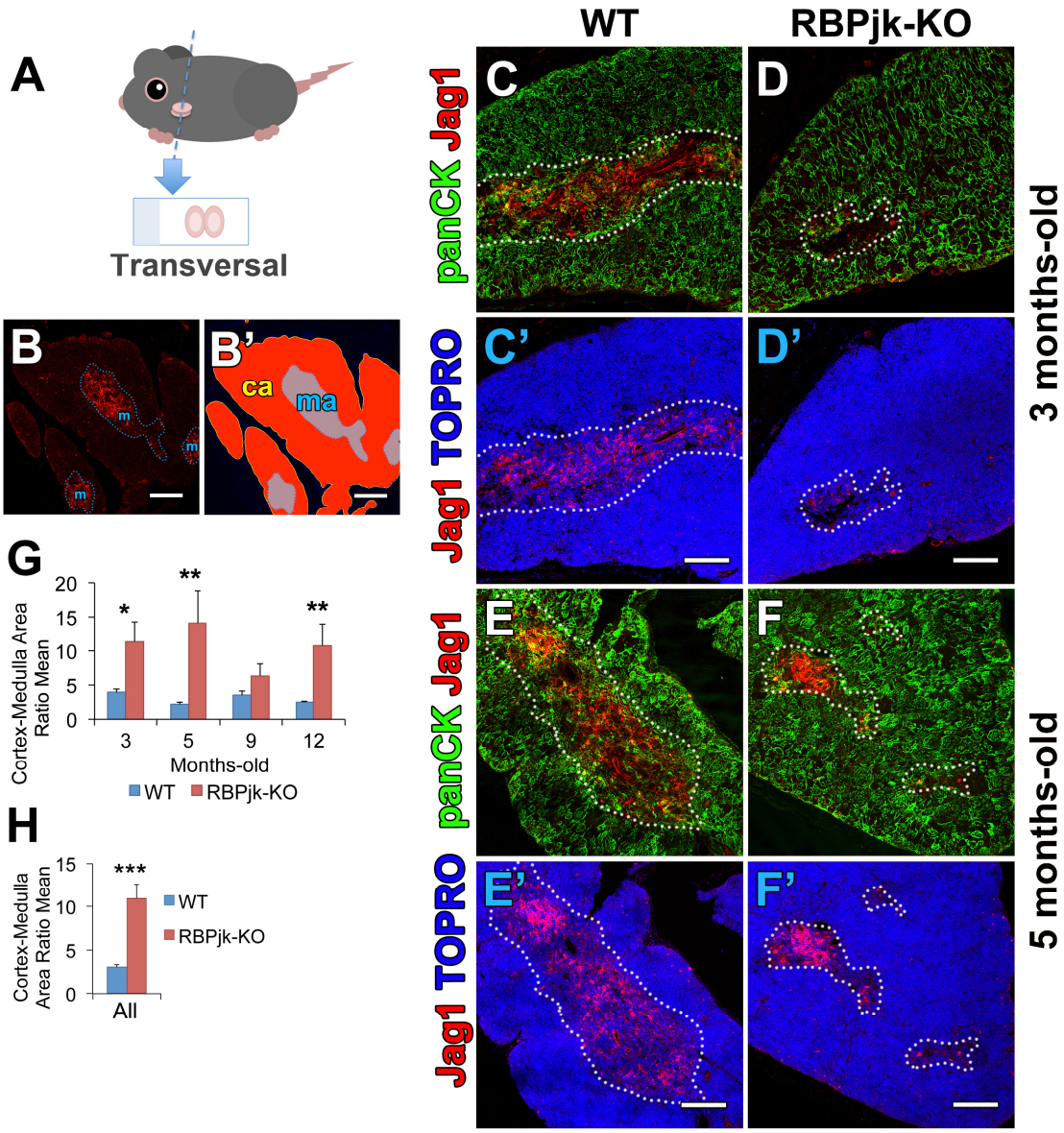


Figure 52. Notch signaling abrogation in TECs severely affects the thymic medulla. Figure shows the histomorphometric study of corticomedullary compartmentalization in WT and RBPjκ-KO^{TEC} postnatal and involuted murine thymus. *A*, Schematic representation of murine thymic lobules orientation for consistent histologic preparations. *B*, Expression of the Notch ligand Jag1 in a WT murine thymic lobule showing Jag1 expression confined exclusively to the medulla. Jag1+ stained area and total thymic area (Topro3 staining) were used to calculate, cortical area (ca) and medullary area (ma) in WT and RBPjκ-KO^{TEC} mice. Scale bar: 200μm. *C, D*, Jag1 and panCK staining of 3 months-old WT and RPBjκ-KO^{TEC} mice thymi, respectively showing a significant reduction in medulla size in the RBPjκ-KO^{TEC} mice. *E*, *F*, Jag1 and panCK staining of 5 months-old WT and RPBjk-KO^{TEC} mice thymi, respectively showing equal results as in *C* and *D*. *Dotted line:* CMJ. Scale bars: 200μm. *G*, Bars graph represents cortical area (ca) and medullary area (ma) ratio mean +/- SEM obtained from 3 (N = 9), 5 (N = 7), 9 (N = 7) and 12 months-old (N = 8) WT thymic lobules, and 3 (N = 12), 5 (N = 7), 9 (N = 6) and 12 months-old (N = 3) RBPjκ-KO^{TEC} thymic lobules. *, p < 0,05; **, p < 0,001. *H,* Bar graph represents cortical area (ca) and medullary area (ma) ratio mean +/- SEM obtained from all WT (N = 31) and RBPjκ-KO^{TEC} (N = 28) thymic lobules analyzed. ***, p < 0,0001.

The average ratio between cortical and medullary areas (C:M Ratio) could be determined from low magnification images (10x) of the thymic tissue stained with cortical and/or medullary markers. Since C:M ration may vary depending on the orientation of the tissue, all thymic samples were consistently embedded and trimmed with the same orientation to allow accurate comparisons (Elmore, 2006). Fig. 52A illustrates murine thymus sampling for cortico-medullary compartmentalization studies. These thymus samples were embedded longitudinally along its antero-posterior axis and trimmed transversally. Resulting tissue sections were stained for the epithelial marker panCK and the Notch ligand Jag1 that, as shown in this study, nicely defines the medullary microenvironment (Fig. 52B). Topro3 staining was used to calculate total thymic area, from which Jag1-defined medullary area was subtracted to obtain the cortical area (Fig. 39B'). Cortical and medullary area measurements were obtained from WT and RBPjκ-KO^{TEC} mice at 3 (N = 4), 5 (N = 3), 9 (N = 3) and 11 (N = 2) months of age, and two images per mice were acquired (10x). Fig. 52C, D and 52E, F show examples of 3 and 5 months-old Jag1-stained WT and RBPjκ-KO^{TEC} thymi, respectively, which revealed a clear reduction of medulla size in RBPjκ-KO^{TEC} mice. In these animals medullary formation and/or homeostasis seems to be affected, as medullary areas are composed by small and discrete islets, similar to those observed at early times of thymic development (Rodewald et al., 2001).

Previous reports showing morphometric measurements of histological sections of rat thymus (Tryphonas et al., 2004), concluded that the average C:M ratio is around 4,8 in up to 3 months-old WT rats (Pearse, 2006). The average C:M ratios calculated for WT mice were similar to that value, as 3 months-old WT mice showed a ratio of 3.93 +/- 0.5, thus supporting the suitability of this histomorphometric method. Conversely, RBPjκ-KO^{TEC} mice showed C:M ratios above 10, which formally prove that Notch signaling abrogation in TECs significantly affect the size of the medullary compartment (Fig. 52G). Given that no significant differences were observed between age groups of mice, mean values of final C:M ratios were calculated considering all mice analyzed regardless their age (Fig. 52H). **In summary, histomorphometric measurements of cortical and medullary thymic compartments demonstrate that selective abrogation of Notch signaling in TECs severely affects the size of the medullary niche.**

4.6. RBPjk-KO^{TEC} thymi host B cells.

Next, we wanted to assess the functional implications of the disturbed medulla in RBPjκ-KO^{TEC} mice. To this end, thymocyte cell suspensions from WT (*N* = 7) and RBPjκ-KO^{TEC} mice (*N* = 10) were analyzed by FACS for expression of T, B and myeloid cell markers. RBPjκ-KO^{TEC} mice had a significantly higher proportion of thymic non-T cells than WT mice (**, p = 0,0093) (Fig. 53A, left). In order to identify the nature of this non-T cell population, cells were stained with the B cell markers CD19 and B220, and the myeloid markers CD11b and Gr1. Electronic gating of the Thy1-negative non-T cell population confirmed that indeed they were B cells, and significantly more abundant within the non-T cell thymic subset of RBPjκ-KO^{TEC} than WT mice (**, p = 0,0011); but no significant differences were found in the proportions of intrathymic myeloid cells (data not shown). Importantly, RBPjκ-KO^{TEC} thymi that host a significant population of B cells showed a significant reduction (**, p = 0,0053) in the frequency of DP thymocytes (Fig. 53C). **In summary, conditional deletion of Notch signaling in TECs has substantial implications for thymus function, that result in the appearance of an important population of intrathymic B cells and the simultaneous reduction of the DP thymocytes compartment.**

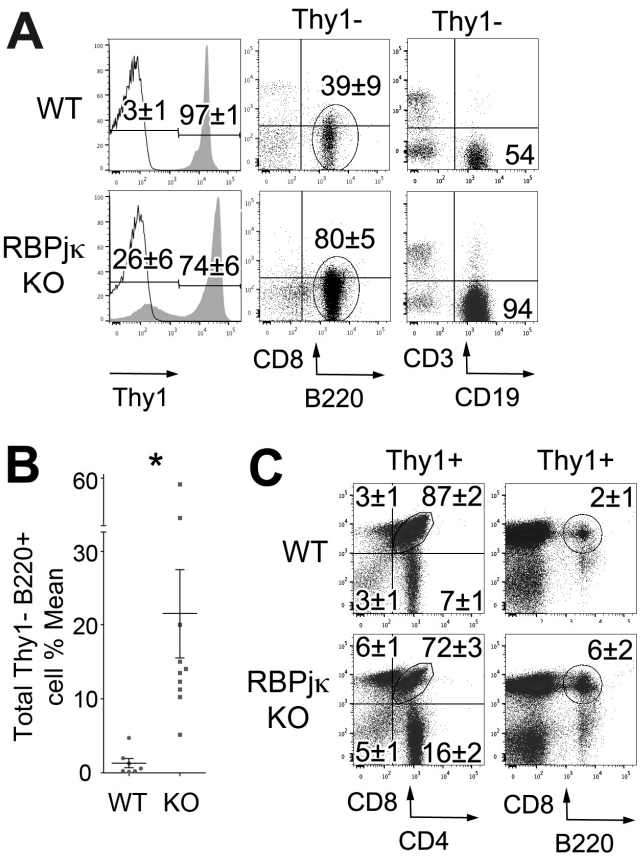
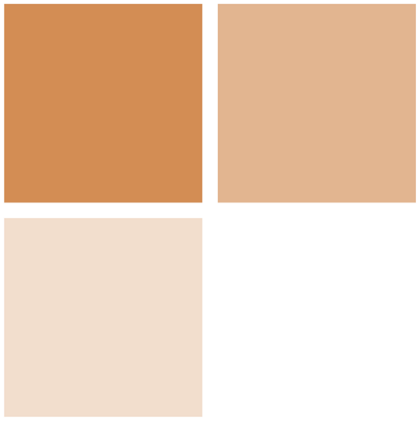


Figure 53. Flow cytometry analysis of WT and RBPjκ-KO^{TEC} thymocyte populations. Mice analyzed were ranging from 3 to 11 months-old. *A, Left,* Thy1- and Thy1+ mean +/- SEM cell frequencies in WT and RBPκ-KO^{TEC} mice. *Right,* Flow cytometry analysis on electronically gated Thy1- cells stained for the B cell markers CD19 and B220 and, T cell markers CD8 and CD3. *B*, Graph representis mean +/- SEM cell frequences of B cells in the thymus of WT and RBPjk-KO^{TEC} mice. *, p < 0,05. C, Flow cytometry analysis on electronically gated Thy1+ cells stained for CD4 and CD8 T cell markers, and the B cell marker B220. Numbers in quadrants show the mean cell frequency (cell %) +/- SEM (WT, *N* = 7; RBPjκ-KO^{TEC}, *N* = 10).



Discussion

Notch ligands and receptors are differentially distributed in the human thymus, defining specific and dynamic niches.

Cellular differentiation is a process imposed by the environment rather than an intrinsic feature of a certain stem or multipotent progenitor cell. In the T-cell lineage, the development of progenitors derived from hematopoietic stem cells resident in the BM is regulated by an specific niche, which is exclusively provided by the thymus. The thymus is an evolutionary conserved organ with a singular microenvironment specialized in generating and "educating" T cells. The understanding of the molecular cues provided by the TME is crucial for developing *in vitro* protocols of T cell generation, and to gain insight on how deregulation of this process can generate hematopoietic malignancies such as T cell leukemia. Most studies on the TME have traditionally focused on TECs, as they are the essential and non-redundant component of the thymic stroma responsible for T cell development (van Ewijk et al., 1994) (Boyd et al., 1993). In mice homozygous for the *nude* mutation (Flanagan, 1966), the thymic epithelial rudiment is unable to further develop into mature cTECs and mTECs (Mori et al., 2010) (Tsukamoto et al., 2005) and, in consequence, these animals lack thymocytes and thymus-derived cells (Wortis et al., 1971) (Vigliano et al., 2011). These data highlight the importance of the signals provided by these highly specialized epithelial cells; but TEC biology is still poorly understood. TEC characterization has been hampered by the fact that TECs are difficult to isolate due to their low numbers and the lack of niche-specific markers. This is crucial since intrathymic T cell development involves the migration of thymocytes throughout different thymic niches composed of particular TEC populations (Petrie and Zuniga-Pflucker, 2007). Additionally, isolation methods destroy the 3D configuration of TECs, which results in the downregulation of specific T-cell inductive signals such as Dll4, and the impairment of TEC function (Mohtashami and Zuniga-Pflucker, 2006). Due to these limitations, we have focused our efforts in characterizing the functional signals provided by TECs and other cell types of the TME in the intact human thymus. The use of immunohistological techniques and confocal microscopy allowed us to identify *in situ* particular intrathymic niches that undergo relevant modifications throughout life, from early ontogeny to postnatal life and later on to age-related involution. Considering the importance of Notch signaling in T cell development, we aimed at characterizing which components of the Notch signaling pathway are expressed by thymic stroma, in order to assess their function at particular T-cell developmental stages. We found that Notch ligands and receptors are differentially distributed in particular niches and are dynamically regulated in a spatio-temporal manner in different stromal cell types, which may explain the complexity of T cell development and the observed age-related changes in T cell output. We show here that certain ligands and receptors define specific niches, such as Jag1 and Notch4, which are exclusively

expressed at the medulla, or Jag2 and Notch3, which are highly expressed at the thymic cortex. However we have discovered some intriguing facts. It is known that the murine thymic cortex hosts 5 of the 6 main T-cell developmental stages (Ladi et al., 2006) (Petrie and Zuniga-Pflucker, 2007), as all immature thymocyte subsets are located in the cortex, while only mature thymocytes reside in the medulla (Lobach and Haynes, 1987). Consequently, the cortex should provide particular niches different enough to support specific developmental steps. Such specificity has been related to the expression of Dll4 (Hozumi et al., 2008); but, surprisingly, Dll4 expression was undetectable in the cortical epithelium of the human postnatal thymus, a finding that concurs with previous data using an alternative experimental approach (Van de Walle et al., 2011). Notch ligands other than Dll4, such as Jag2, are expressed in cTECs, particularly in the CMJ, although the rest of the cortex including the IC and the SCC areas display low expression levels of Jag2 and Dll1. Low Notch ligand expression in the outer cortex is also owed to the low numbers of TECs in this area (Bruijntjes et al., 1993). Therefore, a major cortical area of the human postnatal thymus expresses very low density of Notch ligands.

The scarce expression of Dll4 compared to Jag2 in cTECs may generate some doubts about which ligand induces T cell specification in the human postnatal thymus. In this regard we found that cortical Dll4 expression is restricted to the capillary endothelium, as previously reported (Shutter et al., 2000), and rare subcapsular TECs and thymocytes. This endothelial Dll4 expression may rather suggest that Notch-dependent T-cell commitment is a very early event in human T-cell development occurring prior to, or immediately during, thymus colonization, which is known to happen at the CMJ in the vascularized thymus. The corticomedullary microenvironment includes mesenchymal (MCs) and endothelial cells (ECs), which express virtually all four Notch ligands, especially Dll4. Considering that BM-derived progenitors enter the thymus by leukocyte extravasation through the vascular endothelium (Springer, 1994) of corticomedulary blood vessels, they must encounter Dll4 ligands during this process. Then, endothelial transmigration would localize thymus-seeding progenitors in the perivascular mesenchymal niche, which expresses high Dll4 levels in the human postnatal thymus. Accordingly, we have found ICN1+ hematopoietic cells, likely corresponding with intrathymic progenitors in the human postnatal PVS (data not shown). Likewise CD117+ murine hematopoietic progenitors have been shown to stay in the PVS before entering the thymic parenchyma, indicating that the PVS is the gate from which HPCs enter into the thymus (Mori et al., 2007) and the first thymic tissue they encounter after their egress from the BM. Likewise, early in thymus organogenesis fetal HPCs encounter the mesenchymal microenvironment as soon as they reach the avascular thymic anlage, as they must migrate through the thick mesenchymal capsule surrounding the thymic epithelial

parenchyma (Itoi et al., 2001). The capital importance of the thymic mesenchyme for both T-cell development and TEC differentiation has been extensively studied (Jenkinson et al., 2003) (Itoi et al., 2007), but its function in the postnatal vascularized thymus is still controversial. In addition to the important developmental role of thymic MCs, recent findings showing Dll4 expression in BM MCs points to the BM niche as the first microenvironment presenting Dll4 to thymus-seeding and T-cell lineage priming prior to thymus entry (Yu et al., 2015). Besides Dll4, Jag1 is also expressed in the BM microenvironment (Li et al., 1998) (Karanu et al., 2000), but none of these ligands trigger T-cell development in the BM under normal conditions. In the case of Jag1, this may be due to an intrinsic defect of this ligand to trigger T-cell commitment of very early human HPCs from BM and CB, as shown *in vitro* (Van de Walle et al., 2009), although it supports T-cell development from ETPs (Van de Walle et al., 2013). In addition, the capacity of HPCs to respond to particular Notch-ligands may depend on the regulation of specific Notch receptor expression, as ETPs express mostly Notch1, while BM HPCs predominantly express Notch2, and Notch2-Delta interactions do not allow for T-cell maturation beyond the DN3 (CD25+) stage (Porritt et al., 2004) (Varnum-Finney et al., 1998) (Besseyrias et al., 2007). In this scenario, T-cell specification driven by Dll4 at extrathymic sites could target a particular population of Notch1+ progenitors that differ from *bona fide* BM HPCs and have already egressed from the BM. Sustained Notch1-Dll4 signaling provided during endothelial transmigration and transient residence in the PVS, would then favor thymus colonization and could likely override the lack of Dll4 in the postnatal human thymic parenquima. Intriguingly, this scenario could be different in the human fetal thymus were Dll4 is extensively expressed by cTECs. In fact, our data confirm that Dll4 is downregulated in cTECs in the adult human thymus, as it has been shown in mice (Fiorini et al., 2008), although this downregulation occurs prior to birth in humans, but in the adult thymus (8 weeks-old) in mice. As Dll4 downregulation seems to be mediated by cTEC-DP thymocyte crosstalk (Fiorini et al., 2008), it is possible that the role of Dll4+ cTECs during human thymus ontogeny is restricted to the first third of development, when the full T-cell repertoire and the definitive postnatal thymic configuration are established following colonization at 8.2 weeks (Haynes and Heinly, 1995). Conversely, in the murine thymus all these processes appear to be delayed (Mohtashami and Zuniga-Pflucker, 2006), and Dll4 expression although downregulated, is maintained in postnatal life.

Once Dll4 has induced T-cell fate, alternative signals could support T-lineage progression. Progression of DN2 progenitors to the DP CD3- stage, which is Notch ligand-independent *in vitro* for human DN2-like cells (Taghon et al., 2009), occurs *in vivo* during migration throughout the IC, which displays minor Notch ligand expression. Therefore, as proposed before (Van de Walle et al., 2009), it is possible that decreased Notch signaling is

an early and obligatory event required for appropriate T-cell developmental progression, at least in humans. In this scenario, it can be suggested that the human thymus provides two specific cortical niches, CMJ and IC, distinct enough to support separate cell functions. First, early in development, Notch signaling induced by high expression levels of Notch ligands (likely Dll4) at the thymus entry site (PVS and CMJ) would ensure T-cell commitment of multipotent thymus-seeding progenitors. Later, once T-cell commitment is induced, a decrease in Notch signaling would allow developmental progression of human thymocytes along the αβ T cell lineage; while sustained Notch1 signaling may instead favor γδ T cell development, as shown *in vitro* (Garcia-Peydro et al., 2003) (Van de Walle et al., 2013). In contrast, cortical transmigration of murine thymocytes after T cell fate specification may involve Dll4 signaling continuously provided throughout the IC up to the SCC (Fig. 54). Accordingly, murine αβ T cell development is highly dependent on Notch signaling both *in vitro* and *in vivo,* while such a strong dependence is not seen in humans. The proposed downregulation of Notch activation during human T-cell development may be transient, as Notch signaling is mandatory for β-selection. This occurs at the SCC niche (Ciofani et al., 2004) (Petrie and Zuniga-Pflucker, 2007), where cTECs expressing Dll4 and Jag2, and more significantly Dll1, may fulfill Notch activation requirements of DN3-like developing thymocytes. Accordingly, we found that human thymocytes with active Notch1 accumulate at the CMJ and SCC, while the IC hosts lower numbers of ICN1+ cells. Notably, gene expression studies of microdissected tissue have shown that CMJ and SCC regions in mouse thymus show a unique stromal gene expression profile, suggesting that the IC region may just regulate the trafficking of developing progenitors between those two inductive microenvironments (Griffith et al., 2009). Particularly, Notch activation at the CMJ may control progenitor migration up to the SSC (Maerki et al., 2006) (Mirandola et al., 2012) (Krishnamoorthy et al., 2015), through the IC, and back down into the IC area following β-selection. Apart from Dll4 expression in cTECs, we have seen in the murine thymus a Jag2 expression gradient that is established in the opposite orientation than in the human thymus, with maximal expression levels at the SCC. This differential distribution of Dll4 and Jag2 in murine and human thymus may support the differential requirements of Notch signaling observed for αβ/γδ T cell development between both species, and suggests that specialized populations of cTECs with interspecific characteristics (De Souza et al., 1993) may differentially regulate Notch signaling components in the SCC, IC and CMJ niches (Fig. 54).

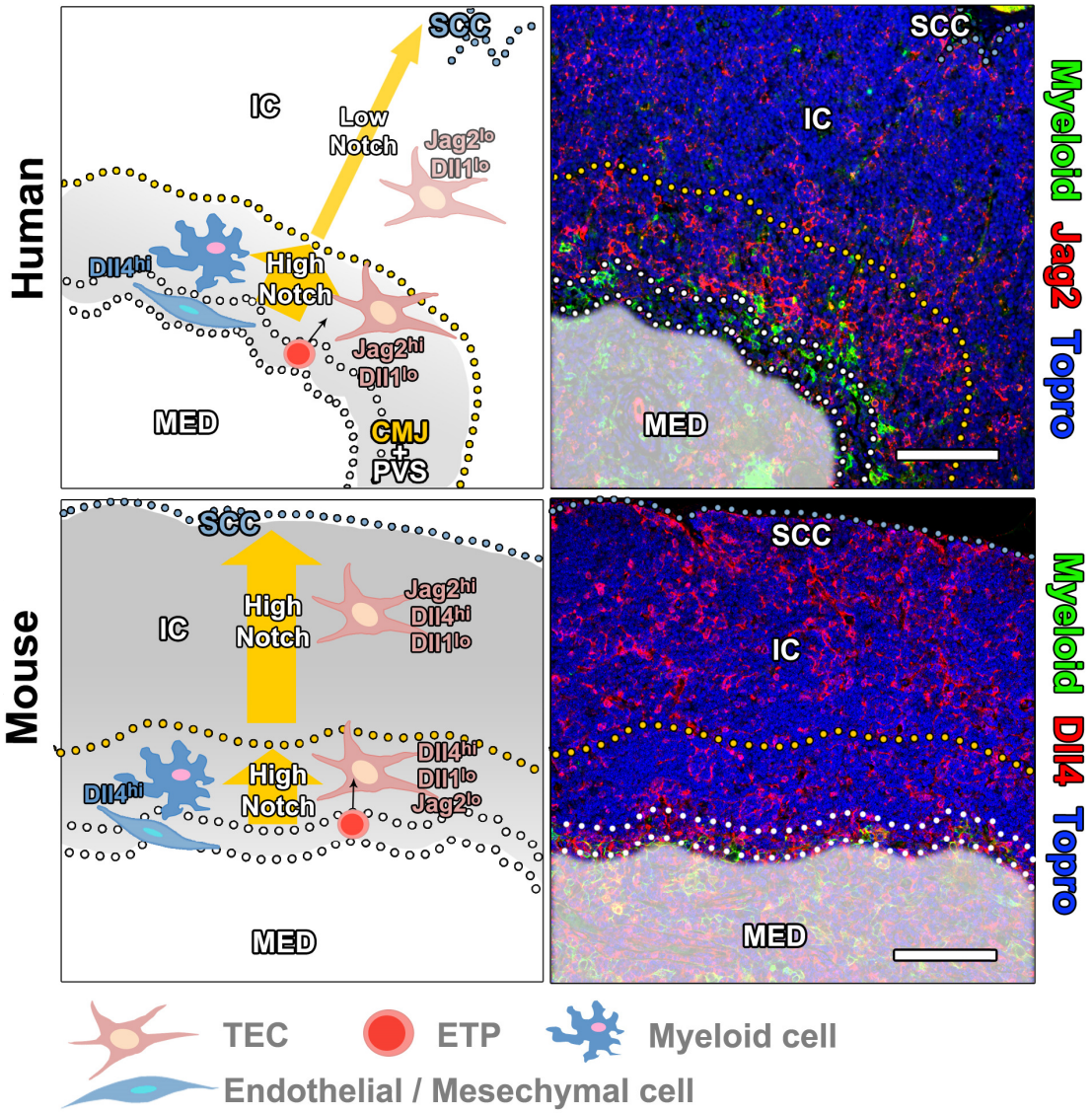


Figure 54. Model of Notch ligand patterning at the postnatal thymic cortex. Left shows and schematic representation of the right confocal image showing myeloid cells and cTECs staining. *Human thymus:* Myeloid cells (green) mainly express Dll4 and Dll1. cTECs (red) express Jag2 and Dll1. As Jag2 expression levels are maximal at corticomedulary cTECs, high levels of Jag2 and Dll4 expression are focused on the narrow region of the CMJ in Jag2-expressing cTECs and Dll4-expressing myeloid cells. *Mouse thymus:* Myeloid cells (green) are also located at the medulla and some at the CMJ. Murine cTECs (red) express Dll1 and Jag2, mostly at the SCC, but they also express Dll4, thus high Noch ligands expression is sustained all along the thymic cortex.

In striking contrast with the low density and diversity of Notch ligands expressed at the thymic cortex, our data pointed to the human thymic medulla as a rich Notch ligand-expressing microenvironment. Jag1 and Dll1 are highly expressed by mTECs, the later mostly by those forming HCs. Importantly, some medullary Dll4-expressing CD11c+ myeloid cells can be found at the CMJ (Fig. 20). Thus myeloid cells provide an important source of Dll4 at the CMJ, where additionally, Jag2 is highly expressed by cTECs. Therefore, following Notch activation induced by Dll4-expressing endothelium and PVS, Notch signaling could be further sustained at the CMJ by Jag2-expressing cTECs and Dll4-expresing myeloid cells.

Additionally, our study reveals that postnatal configuration of the human TME is not steady. Many events as age, pregnancy, infections, stress, may influence the TME and thus modify the T cell outcome, but age is the main trait to battle against when facing poor immune function in healthy individuals. It has been long thought that the aged thymus loses its ability to generate T cells. However it has been shown that the remaining thymic parenchyma is normally functional in the involuted thymus (Flores et al., 1999). Undoubtedly age-related histologic changes, like PVS enlargement and adipose tissue deposition, are the main remodelers of intrathymic niches as both diminish the volume of functional parenchyma. But we have found that aged TECs actively regulate Notch ligand expression in particular thymic niches, similarly to what has been described in the fetal thymus. We found that Jag1 is specifically upregulated at the SCC, and Jag2 downregulates is particularly enriched expression at the CMJ. This active remodeling might be viewed as one of the causes of poor T cell generation in the aged thymus, but it can also be view as a compensatory mechanism that arises in the remaining functional parenchyma when thymic structure is lost. **Altogether, our expression data demonstrate that Notch ligands and Notch receptor expression is dynamically regulated in a spatio-temporal manner in human TECs. This regulation fulfills the sequential Notch signaling requirements of developing thymocytes, although such requirements vary between mice and humans according to the interspecific diversity of TME.**

Notch ligands differentially influence αβ and γδ T cell development.

Notch ligands differentially regulate cell fate decisions in diverse developmental systems. In the hematopoietic system, Delta ligands are involved in T cell commitment and T lymphocyte peripheral maturation, while Jagged ligands are involved in γδ and NK lymphocytes development and peripheral functional T cell differentiation (de La Coste and Freitas, 2006). Likewise, it is well established that Notch signaling regulates the αβ versus γδ T cell lineage choice (Ciofani and Zuniga-Pflucker, 2010) (Kreslavsky et al., 2010) (Taghon et al., 2012), besides the contradictory results between mice and humans found regarding the signal strength required for such developmental decision (Washburn et al., 1997) (De Smedt et al., 2002) (Garcia-Peydro et al., 2003) (Garbe and von Boehmer, 2007) (Van de Walle et al., 2009). The identity of the Notch ligands regulating this developmental choice *in vivo* remained unclear. Here we demonstrate that differential Notch ligand-induced signaling control human αβ and γδ T cell development and, show that Jag2 has an unique ability in promoting almost exclusively the γδ T cell lineage at the expense of the αβ lineage, which is only supported by Dll1, Dll4 and Jag1 ligands. Conversely, Jag1 is equally efficient

in generating cells of both, αβ or γδ T cell lineages, with a clear impairment of the γδ T cell lineage when compared with Jag2, which acts similarly as Delta ligands in γδ T cell specification, as previously suggested to occur for T cell fate specification on human CD34+ CB progenitors (Van de Walle et al., 2011) and later confirmed for αβ/γδ-lineage specification on ETPs during the development of this thesis (Van de Walle et al., 2013).

Although confirming a differential role for Notch ligands in αβ and γδ T cell development, the data obtained by *in vitro* co-cultures coming from bulk populations, cannot discriminate whether differential Notch signaling directly influence the αβ/γδ T-cell fate decision in uncommitted progenitors, or rather it affects the survival and expansion of lineage-restricted pre-committed thymocyte populations, or both. Taghon and colleagues (Van de Walle et al., 2013) approached this issue by single cell co-culture of human intrathymic progenitors onto the different OP9 ligand-expressing stromas. These clonal analysis allowed them to conclude that Notch ligands differentially influence αβ/γδ cell fate decision, as Dll4-seeded wells recovered both αβ and γδ lineages, whereas Jag1-seeded wells almost exclusively yield αβ T cells and Jag2 mainly recovered γδ T cells. Nevertheless, in order to avoid Notch ligand-mediated effects in T cell commitment, the progenitors used in these clonal experiments were CD34+ CD1a+, corresponding with late DN2-like progenitors in which αβ/γδ T cell commitment may have been occurred already, as illustrated by several references (Prinz et al., 2006) (Rothenberg, 2014) (Res and Spits, 1999). Thus clonal analysis using early DN2-like lymphoid progenitors, in which T but not αβ/γδ cell fate has been specified, as the CD34+ CD1a- cells used in our experiments, would be useful to evaluate the putative instructive role of Notch ligands in αβ/γδ T cell fate determination. In this line of arguments, further analysis of the mechanisms involved in αβ T cell generation in our cultures, showed that Jag2 promotion of the γδ T cell fate at the expense of the αβ T cell fate, was due in part, to the specific impairment of the generation of a icTCRβ+ thymocyte population. Taghon and colleagues (Van de Walle et al., 2013), easily approached the question whether Jag2 negatively impact αβ lineage decision or affect the survival and/or expansion of the icTCRβ+ population, by transducing a TCRβ chain into intrathymic hematopoietic progenitors later co-cultured in the presence of Jag2. TCRβ transgene rescued the Jag2-mediated impairment of the αβ T cell linage, thus demonstrating that Jag2 is not affecting the survival and/or expansion of icTCRβ+ αβ-lineage cells, but specifically impairing its generation by blocking TCRβ chain expression. We decided to approach the issue of an inductive versus selective role of Notch ligands in αβ/γδ lineage commitment by gene expression analyses on T cell committed αβ/γδ uncommitted DN2-like progenitors "primed" during 6 days of co-culture with the different Notch ligands. During those 6 days of

co-culture, T cell progenitors did not acquired TCR expression, neither intracellular nor at the cell membrane. We assayed a narrow set of 7 known αβ/γδ lineage-specific genes, and non of them were specifically expressed by γδ T cells and regulated under differential Notch signaling, thus suggesting that Jag2 specific promotion of the γδ T cell lineage is not specifically mediated by known γδ-specific transcription factors such as Sox13 or Etv5 (Melichar et al., 2007) (Jojic et al., 2013) (Malhotra et al., 2013). However, these results do not exclude the possibility of an inductive role of Jag2 by other transcription factors not yet characterized. Precisely, the characterization of human αβ/γδ lineage-specific transcription factors involves certain difficulties. Several studies in mice (Jojic et al., 2013) (Mingueneau et al., 2013) have shown that almost all the factors known to provide crucial functions for αβ or γδ lineage specification, are also vital for early T cell commitment. Nonetheless, some factors are specifically maintained by each lineage after T cell fate specification (Novershtern et al., 2011). Conversely, similar studies conducted on human HPCs found a substantial mixed use of factors by αβ and γδ lineages, thus hampering the characterization of a single factor responsible for αβ or γδ T cell fate determination (Novershtern et al., 2011). Nevertheless, our data show that *TOX2* expression was significantly increased by Jag2 and Dll4 ligands, which both induce strong Notch signaling activation needed for γδ T cell fate specification (Van de Walle et al., 2009). Tox2 is a transcriptional activator member of the thymocyte selection-associated high mobility group box protein family. Its cognate member family, Tox1 has been shown to play key roles in the development of lymphoid tissue inducer cells (LTi), CD4 T cells and NK cells (Aliahmad and Kaye, 2008) (Aliahmad et al., 2010), and Tox2 has been shown to control NK development from CB-derived HPCs by regulation of T-bet expression (Vong et al., 2014), a transcription factor involved in γδ T cell functional maturation (Ribot et al., 2014). Thus *TOX2* might be involved in early γδ T cell differentiation although later downregulated upon TCRγδ expression. In order to shed light about other mechanism/s responsible for the differential role of Jag2 in γδ T cell development, we studied intrathymic gd T cell differentiation *ex vivo* and *in vitro* with the four different Notch ligand-expressing OP9 cells. As detailed at the introduction, human but not murine γδ T cells may follow a DP developmental pathway. DN, DP and CD4+ and CD8+ SP γδ T cell populations can be found in the human thymus. Additionally, we may divide γδ thymocytes in three populations according to their different expression levels of CD1a (high, dim and negative). CD1a+ γδ thymocytes express RAG-1 recombinase and do not respond to TCR engagement, thus constitute an immature population exclusively found in the thymus (Offner et al., 1997) which exclusively corresponds with the DP and CD4+ phenotypes *in vivo* and in *vitro*, thus positioning the DP and CD4+ γδ thymocyte populations prior the DN and CD8+ ones. A minor population of CD1a+ DN γδ thymocytes (less than 10% of total DN γδ thymocytes) can be also found, which may correspond with γδ T cell progenitors as previously suggested (Van

Coppernolle et al., 2012). Intriguingly, the frequency of the CD1a+ DP γδ population, underrepresented *in vivo*, was the most abundant γδ population obtained *in vitro*, specially in OP9-Jag2 and OP9-Dll4 co-cultures, thus suggesting a role for strong sustained Notch activation in the generation of these immature γδ T cell populations. Indeed, one of the mechanisms responsible for Jag2 support of the γδ T cell lineage, was the specific promotion of immature γδ populations. This singular ability of Jag2 in promoting the generation of CD1a+ γδ thymocytes *in vitro* was not unique of Jag2, as under Dll1 and Dll4 signaling, DP and CD4+ γδ T cells were also promoted when compared with Jag1, but much less efficiently than with Jag2. It is quite intriguing that in mice γδ T cells do not follow a DP pathway, although they also follow different maturation stages defined by CD24. This may lead to think that human DP and CD4+ γδ T phenotypes might be αβ-lineage thymocytes that were switched to the γδ-lineage by an early strong TCR or preTCR signal, as predicted by the "signal strength" model of αβ/γδ lineage determination. To investigate this possibility, intracellular expression of the TCRβ chain was analyzed in DP and CD4+ γδ T cells generated *in vitro* with the different Notch ligands. We found no expression of the TCRβ chain by any TCRγδ+ cell in any culture condition, result supported by icTCRβ expression analyses in the bulk population. Thus CD4+ and DP human γδ developmental stages are true γδ-lineage thymocytes. Opposing to Jag2, Jag1 was clearly impairing the generation of these two immature populations of γδ thymocytes, although it was the only ligand allowing CD1a downregulation in the DN and CD8+ γδ populations. It has been shown that absence of Notch signaling drives CD1a+ DN γδ progenitors towards the CD1a- DN or CD8+ stage without passing through the CD4 and/or DP stage (Van Coppernolle et al., 2012), thus we cannot account for CD1a downregulation as an specific inductive effect of the Jag1 ligand, or just a consequence of its weak ability in activating Notch signaling in thymocytes. In this way, Jag1 signaling might not be strong enough to drive γδ T cell progenitors towards the DP differentiation pathway thus allowing a straightforward generation of DN and CD8+ γδ T cells from the γδ progenitor and/or favoring the maturation of CD1a+ DP / CD4+ γδ thymocytes. In order to clarify this issue, and confirm the progenitor-progeny relationship of CD1a+ and CD1a- gd thymocytes, *in vitro* γδ T cell differentiation assays were performed using *ex-vivo* isolated human $CD1a^{hi}$ γδ thymocytes, which included CD4+, DP and a minor population of DN γδ T cell progenitors. This assays confirmed two opposite roles for Jag1 and Jag2 in γδ T cell development, as Jag1 allowed CD1a+ γδ thymocytes maturation towards a CD1a- stage, in a similar way as absence of Notch signaling (OP9-GFP) does, although maintaining a small population of CD1a+ γδ T cell thymocytes that may correspond with CD1a+ DN γδ T cell progenitors. Conversely, Jag2-mediated Notch signaling constituted a strong proliferative signal for CD1a+ γδ thymocytes which inhibited their differentiation towards the CD1a- developmental stage. In this way, Jag2 was less efficient than Jag1 in

generating CD1a- mature γδ thymocytes, as Jag2 co-culture generated 4 times more γδ T cells than Jag1 but yield equal number of mature CD1a- γδ T cells, thus confirming previous findings suggesting that generation of CD1a- DN / CD8+ γδ T cells is independent of Notch signaling (Van Coppernolle et al., 2012), and suggesting that CD1a- γδ T cells generated on OP9-Jag2 co-cultures derive from cells that initially might have escaped from Jag2 ligand engagement. Jag2-mediated effect on immature CD1a+ γδ thymocytes perfectly illustrates that proliferation and differentiation are mutually exclusive processes. In this regard, Jag2 resembles a typical positive selection signal that induces proliferation and survival of a certain population TCR-expressing thymocytes. We know that αβ-lineage cells follow two successive checkpoints that result in programmed cell death if not fulfilled. The fist one, β-selection, is a process in which productive rearrangements of the *TCRB* locus and subsequent membrane pre-TCR expression results in extensive proliferation, differentiation into DP CD3+ preT cells and creates TCR diversity, as each daughter cell generates an unique TCR-α chain, resulting in daughter cells that each contains a different αβ-TCR despite sharing the same TCR-β chain. This process has not been observed in γδ T cells, which express both TCR chains at the same time. Next, TCRαβ-expressing DP thymocytes are engaged by low affinity interactions with self-MHC complexes that rescue them from apoptosis and induce again their survival and proliferation. But the developmental checkpoint in which successful γδ T cell receptor (TCR) gene rearrangements and/or MHC-self affinity are controlled during γδ T cell development are poorly characterized in humans. Studies in mice have shown that after TCRgd expression some DN3 γδ thymocytes selectively express CD27, which is upregulated in αβ-lineage thymocytes after β-selection (Taghon et al., 2006). Additionally, TCRb-/- mice have shown several DN thymocyte subsets enritched with in-frame TCRδ rearrangements, thus suggesting that cells with TCRδ in-frame rearrangements have a selective advantage, although there is no evidence of the involvement of a "preTγ" chain in this process. Nevertheless, TCRg expression do not results in proliferation, although is absolutely required for the generation of DP CD3- thymocytes (Passoni et al., 1997). Conversely, signaling through the γδ TCR induce a discrete proliferative phase of much less extend to that induced by the preTCR (Taghon et al., 2006) (Prinz et al., 2006). Interestingly, LAT-deficient mice are not able to generate mature γδ T cell (Nunez-Cruz et al., 2003) (Prinz et al., 2006), thus suggesting that some TCRγδ-mediated mechanism of positive selection is needed for normal T cell development (Wells et al., 1993). In this regard, Jag2-mediated signaling may act cooperatively with the γδ TCR on immature thymocytes inducing proliferation and thus allowing the expansion of positively selected immature γδ thymocytes. The differential signal strength delivered by the different Notch receptor - Notch ligand pairs may explain the differential outcome observed in γδ T cell development, as proposed for αβ/γδ T cell specification (Abe et al., 2010) (Van de Walle et al., 2013). Jag2 induces the strongest Notch

signal in human intrathymic progenitors, but not in CB HPCs. This was explained because the strongest Notch activation in CB HPCs is mediated through Notch1-Dll4 pair (Van de Walle et al., 2011), while on intrathymic progenitors the strongest signal is mediated through the Notch3-Jag2 receptor pair (Van de Walle et al., 2013). Likewise, it has been proposed that Notch1-Jag1 signaling do not favor γδ T cell development because the resulting Notch1 activation is not strong enough to upregulate the Notch1 target gene *NOTCH3* and thus avoid further strong Notch3-Jag2 γδ-inductive signal. But this argument opposes several findings as *in vivo*, T cell lineage specification occurs by Dll4, a strong activator of the Notch1 receptor. Additionally, our *in vivo* expression data show broad Notch3 expression in cortical αβ-lineage thymocytes finding consistent with Notch3 rapid upregulation in thymocytes after Notch1-Dll4- mediated T cell commitment (Felli et al., 1999) (Van de Walle et al., 2009). Thus or data suggest that Notch1 activation accomplished *in vivo* on ab/gd uncommitted intrathymic progenitors is strong enough to upregulate Notch3 expression in subsequent developmental stages. Likewise, we show that Notch1 is active in CD34+ intrathymic progenitors and that Notch1 receptor is the most prominent receptor expressed by human γδ thymocytes, specifically at the DP stage, a finding in line with previous data from our laboratory and others (Van de Walle et al., 2009) (Martin-Gayo, E. et al. *submmited*). Altogether, these data suggest that Notch1 might play an important role in ab/gd T cell fate determination. Anyhow, additional regulatory mechanism to that proposed by Thagon and collegues might be acting *in vivo* in the human thymus, as a model based in specific Notch receptors – Notch ligands interactions may be too simplistic. There are many regulators that may act in the human thymus to modulate Notch signaling strength. Notch signal strength has been traditionally estimated by the level of transcriptional activation of Notch target genes (Van de Walle et al., 2009) (Gonzalez-Garcia et al., 2012), which may be a direct consequence of three things: (1) the number of intracellular Notch domain molecules (ICN) cleaved; (2) the number of ICN molecules reaching the nucleus, and (3) the half-life of ICN-RBPjk-MAML-DNA complexes (Liu et al., 2015). These factors may vary, for example, by ubiquitination of ICN or Fringe-mediated glycosylation of Notch receptors, which potentiate Notch1 signaling through Delta ligands and Jag2, but not through Jag1 (Van de Walle et al., 2011). Additionally, other morphogenetic signaling pathways, as the Wnt pathway, might be operating in the thymus in specific TMEs (data not shown). For example, it has been demonstrated that secreted frizzled-related proteins (SFRPs) may bind ADAM10/17 metalloproteases and downregulate its activity, thus interfering with Notch signaling pathway activation (Esteve et al., 2011). Additionally, ligand lateral mobility, orientation and density modulate receptor activation levels, as limiting the lateral mobility of Dll4 displayed on lipid bilayers, enhances Notch1 activation (Narui and Salaita, 2013). Thus the expression of the same ligand in different cell types does not necessarily must account the same Notch activation threshold in the

responding cell. However, and based in our findings, we can grade Notch ligand-induced signals delivered to DN2-like progenitors in the following order: Jag2, Dll4, Dll1 and Jag1, and we can say that the opposite functional outcomes of Jag2 and Jag1 ligands correlates with their opposite location in two different functional thymic niches, the cortex and the medulla, respectively. **Thus our findings establish that the equilibrium between two Notch ligands with distinct spatial expression patterns in vivo, and opposing functional roles in vitro, may regulate γδ T cell development,** in a similar way to that described for other Notch-dependent tissues (Benedito et al., 2009).

Role of Notch signaling in functional differentiation γδ T cells.

At the light of recent findings indicating that γδ T cell maturation occurs in the murine thymus (Narayan et al., 2012), we decided to investigate if human γδ T cell functional maturation equally occurs at the TME and if its influenced by differential Notch signaling. In mice, numerous reports point to Notch signaling as a molecular cue involved in the development of IL-17-secreting γδ T cells (Shibata et al., 2008) (Ribot et al., 2009) (Michel et al., 2012) (Mukherjee et al., 2009), while INFγ-secreting cells are generated after TCRγδ engagement (Jensen et al., 2008). We were not able to detect a significant population of IL17-secreting γδ T cells neither on PMA/ionomycin-stimulated ex-vivo-isolated human γδ T cells nor in γδ T cells generated in our co-cultures with OP9 Notch ligand-expressing cells (data not shown), thus discarding the idea of finding naturally occurring γδ Th17 cells in the human thymus. Nevertheless, differential Notch signaling provided by the TME may regulate the generation of different functionally pre-programmed γδ T cell subpopulations. Thus *in vitro* generated γδ T cells were activated with plate bound anti-CD3 and IL-2. Although regular αβ Th17 cells were easily detected in human peripheral blood samples, thus discarding possible experimental issues during cytokine labeling, IL17 γδ T cells were not detected. We cannot discard the possibility that our activation conditions skewed Th17 potential, as IL2 is a typical IFNg-polarizing cytokine (Manel et al., 2008). Conversely to γδ IL17 cells, we were able to detect an efficient generation of INFγ gδ-secreting cells, especially in OP9-Jag1 co-cultures. It has been shown that IFNγ-secreting cell mostly correspond with the Vδ2Vγ9 peripheral blood phenotype, while IL-17-secreting γδ T cells correspond with tissue Vδ1 subsets. Therefore the favored generation of IFNγ+ γδ cells in relative terms obtained in Jag1 co-cultures, might be the consequence of the defective support of CD1a+ immature Vδ1 and Vδ1-/Vδ2- γδ subsets, which are both efficiently supported by Jag2 and Delta ligands. The identity of those Vδ1-/Vδ2- γδ T cells remains obscure as no suitable antibodies have been developed yet to detect the up to 6 putative Vδ chains identified in humans. Another interesting possibility is they may correspond with γδ T regulatory cells, as Vδ1+ PB γδ T cells when stimulated *in vitro*

with anti-Vδ1 acquire regulatory features as Foxp3, CTLA-4 and TNFR expression and produce TGFb1 (Hua et al., 2013). Further characterization of these γδ T cell populations with T regulatory cell markers such as Foxp3, Galectin1, CTLA-4 or GARP (Roncarolo and Gregori, 2008) (Battaglia and Roncarolo, 2009) would be useful. Nevertheless, the information obtained about the molecular cues involved in the regulation of human γδ T cell development, is extremely valuable for implementing new protocols of *in vitro* γδ T cells generation. Only one reference in the literature address the generation of γδ T cells in vitro from human HPCs (Groh et al., 1991), and recent approaches of large scale generation of human γδ T cells in vitro have been focused in expansion of peripheral blood γδ T cells which are mostly of the Vδ2Vγ9 subtype. Conversely, our data show that co-culture of intrathymic human hematopoietic progenitors with genetically-modified stromal cells for the expression of Notch ligands, specially Jag2, generate great numbers of diverse Vδ γδ T cell subsets. A single DN2-like progenitor may give rise about 300 γδ T cells showing the same Vδ diversity as found *in vivo* in the human thymus. Unfortunately, likewise within the thymus, only 10% of those γδ T cells are mature, as the precise mechanism exerted by Jag2 consists in promoting the proliferation of immature CD1a+ DP and CD4+ intermediate gd T cell differentiation stages. These immature γδ T cells are not functional as they do not proliferate after TCR engagement (Offner et al., 1997). But maturation efficiency can be boosted by sequential Notch signaling deprivation and TCR engagement with plate bound anti-CD3 antibodies and polarizing cytokines, obtaining mature CD1a- DN/CD8+ γδ T cells which are diverse regarding Vδ expression and fully functional as they secrete INFγ. **Thus, the *in vitro* co-culture system describe here may serve for large-scale generation of human γδ T cells and overcomes the limitations of current protocols of γδ T cell generation for adoptive transfer of ex-vivo isolated in-vitro expanded peripheral blood human γδ T cells. New xeno-free *in vitro* approaches, as Jag2-rich hydrogels, could be the ultimate approach to large-scale generate in vitro functionally diverse populations of γδ T cells suitable for the clinic.**

Gamma-delta T cell intrathymic dynamics.

In contrast to the well-defined sequential migration that αβ-lineage cells follow within the TME, intrathymic dynamics of γδ T cells are poorly understood. We have found that human γδ T cells are more abundant at the cortex, where they preferentially accumulates at the CMJ. Previous studies (Groh et al., 1989) (Falini et al., 1989) reported that γδ T cells are more abundant at the medulla than the cortex, but this discrepancy can be explained by a miss definition of the cortico-medulary boundary in the absence of epithelial markers. The differential distribution of immature and mature γδ T cells found in the cortex and the

medulla, suggests that developmental progression of γδ thymocytes from the cortical immature CD1a+ to the medullary mature CD1a- stage might be accompanied by cortico-medulary transmigration and thus membrane expression regulation of chemokine receptors. Nevertheless, this possibility do not exclude that a proportion of mature CD1a- γδ T cells may be generated directly at the thymic medulla by the Notch-independent pathway described by Vandekerckhove and colleagues (Van Coppernolle et al., 2012), and observed in our γδ T cell co-cultures. As previously reported (Ueno et al., 2004) (Misslitz et al., 2004), cortico-medulary transmigration may be regulated by CCR7, as medullary TECs express the CCL19 and CCL21 chemokines. Lack of a suitable CCR7 antibody frustrated our effort in detecting CCR7 in γδ thymocytes, although we cannot exclude the possibility that density gradient preparations (Ficoll) of thymocyte suspensions decreased chemokine receptors expression as previously demonstrated (Nieto et al., 2012) (Berhanu et al., 2003). Due to this inconvenient, transwell migration analyses with CD1a+ and CD1a- γδ thymocytes would be capital to properly assess differential regulation of chemokine receptors expression in both γδ T cell populations. Despite these limitations, we were able to detect CXCR4 expression in αβ-lineage and γδ thymocytes. CXCR4 was specifically expressed in DP and CD4 immature γδ thymocytes, suggesting that location of immature γδ thymocytes at the CMJ, might be mediated by the CXCR4 chemokine receptor. Nevertheless, only 50% and 30% of DP and CD4+ γδ thymocytes expressed CXCR4, respectively. These differences on CXCR4 expression within both populations suggest that DP and CD4 γδ thymocytes may regulate CXCR4 expression in a certain point of development as a consequence of events like positive selection, as demonstrated for CCR9, which is up-regulated in CD69+ DP αβ post-selected thymocytes (Uehara et al., 2002). Indeed, in αβ T cells positive selection correlates with cortico-medulary transmigration of positively selected thymocytes into the medulla, while non-selected DP αβ-lineage thymocytes remains moving randomly at the cortex where they die by neglect. Similarly, CXCR4 acts as a co-stimulator during β-selection of αβ-lineage cells by regulating the steady-state localization of immature thymocytes at the SCC, thus facilitating delivery of pre-TCR-induced survival and proliferation signals (Trampont et al., 2010). In this line of arguments, and as discussed earlier, Jag2-mediated Notch signaling may be acting cooperatively with the γδ TCR during a putative positive selection checkpoint at the CMJ and, in a similar way to αβ T cells, γδ T cell positive selection may induce the migration cortical immature CD1a+ γδ thymocytes into the medulla, thus avoiding putative overproliferation by sustained Jag2 signaling at the CMJ. **In summary, intrathymic progenitors receiving the T cell commitment signal by Dll4 at extraparenchymal sites, such as the endothelium or the PVS, and/or by Jag2 at the CMJ, rapidly move upwards the cortex, where low Notch ligands density allow αβ-lineage progression. Conversely, sustained Jag2 signaling at the CMJ would favor γδ T cell generation by selective**

induction of γδ-lineage commitment and specific support of intermediate immature γδ populations. The time length that an immature γδ thymocyte stays at the CMJ must be strictly limited to avoid the overproduction of immature γδ T cells mediated by Jag2. This mechanism may involve a γδ positive selection like process which regulate the CXCR4 receptor expression, thus allowing cortico-medulary migration into the thymic medulla (Fig. 55). Nevertheless this scenario may have an objection derived from the fact that there is no other phenotypic marker than the TCRγδ itself for tracking γδ lineage-committed thymocytes. Consequently, it is impossible to characterize early intermediate stages of γδ T cell differentiation between the uncommitted DN2-like progenitor and the γδ-committed thymocyte. Thus γδ T cell stages prior TCRγδ expression cannot be characterized neither located in vivo. Because of this, we cannot exclude that cortical immature CD1a+ γδ T cell progenitors, after γδ T cell commitment and prior TCRγδ receptor expression, migrate from the CMJ to the SCC and acquire CD4, CD8 expression on their way up to the SCC in a similar ways as αβ T cells do. Upward migration from the CMJ to the SCC might serve as well as a mechanism for avoidance of Jag2-induced γδ T cell progenitor proliferation at the CMJ. Nevertheless, migration to the to the medulla is more likely, as Jag2 is much less expressed there than at the cortex, and because γδ T cells do not follow a β-selection-like process as αβ T cells do (Passoni et al., 1997). In order to avoid this technical limitation in the detection of early steps of γδ-lineage determination, the group of Malissen developed a mouse model expressing the GFP reporter gene knocked into the *TCRD* constant region gene (Prinz et al., 2006). They found that the *TCRD* locus becomes transcriptionally active at late DN1 stage; unfortunately they do not show the *in vivo* location of those late DN1 GFP+ cells. Nevertheless, transcriptional activation of the *TCRD* gene do not necessarily means γδ T cell commitment, which has been defined to occur at late DN2 stage (Rothenberg, 2014). Additionally, considering that (1) human γδ T cells follow a DP differentiation pathway while murine γδ T cells do not, and (2) the differences between the murine and human TMEs exposed in this work, we can not assume similar human and murine γδ T cell dynamics. The ultimate assessment of γδ T cell dynamics would be performing real time imaging of human intrathymic progenitors transduced with ICN1 on FTOC or intact human thymic lobules, as ICN1-expressing human intrathymic progenitors adopt the γδ T cell fate in FTOC (Garcia-Peydro et al., 2003).

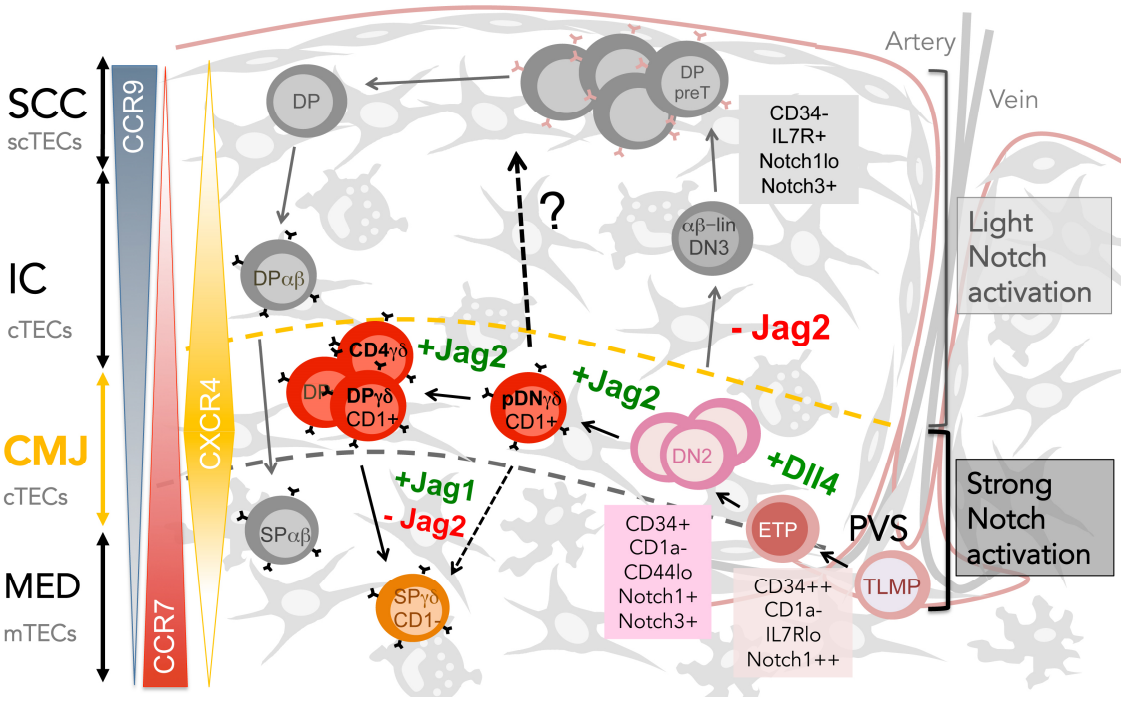


Figure 56. Model of γδ T cell intrathymic dynamics. Schematic representation of αβ (grey) and γδ-lineage (colored) T cell intrathymic dynamics. Colored ligand legends indicate: *red:* impairment (-); *green:* promotion (+). Colored bars at the left indicate chemokine receptors (and corresponding ligands) gradients according to literature. Right grey boxes indicate Notch signaling activation according to Notch ligands expression levels and density. TLMP: Thymic lymphomyeloid progenitor; ETP: Early thymic progenitor; pDN γδ: double-negative γδ T cell progenitor.

Notch signaling in intrathymic crosstalk: the role of Notch in TEC biology.

TECs are the stromal component of the thymus specialized in T cell development support. They are divided in two specialized subsets, which differ in their anatomical location, cellular morphology and functional properties. Both mTECs and cTECs express common markers but can be distinguished by differential expression of cytokeratins and other markers (Gray et al., 2006). Despite their differences, cTECs and mTECs originate from a common epithelial cell progenitor (TEPC) (Rossi et al., 2006) that persists in the postnatal thymus (Bleul et al., 2006) and contribute to the maintenance of the steady-state thymus, at least in mice. The existence of this bipotent epithelial progenitor in the human postnatal thymus is pending to be determined, mainly due to the absence of specific markers to unequivocally characterize these progenitors. TEC development is divided in two phases: an early thymocyte-independent differentiation stage, which drives the initial specification of the bipotent TEPC into the cTEC and mTEC lineages; and a late thymocyte-dependent phase, initiated in later phases of gestation, involving terminal differentiation of cTECs and mTECs. Several signaling pathways, as TNFR and LFβR, have been shown to be involved in the second thymocyte-dependent phase of TEC development (Boehm et al., 2003) (Rossi et al., 2007) (Akiyama et al., 2008) (Hikosaka et al., 2008). However, the signaling requirements for

the early phase remain obscure. Foxn1 is an essential factor during this early thymocyte-independent phase of TEC development (Klug et al., 2002) (Jenkinson et al., 2005), and continuous expression of FoxN1 is required throughout adulthood to maintain the functional capacity of TECs (Chen et al., 2009) (Cheng et al., 2010) (Tsukamoto et al., 2005). Interestingly, in the skin Foxn1 is needed for Notch1 receptor expression and hair shaft differentiation (Cai et al., 2009), but no such relationship of Notch1 and Foxn1 have been found in TECs. Nevertheless, TECs express Notch ligands and Notch receptors from very early in development in mice and humans, thus TECs may provide each other Notch signaling cues. Two capital checkpoints of TEC biology could be regulated by Notch according the two phases of TEC development accounted earlier: (1) maintenance and renewal of the TEC progenitor cells (TEPCs) and cTEC versus mTEC fate specification, and (2) terminal TEC differentiation, homeostasis and senescence during postnatal life. Only one report (Masuda et al., 2009) has indirectly addressed the role of Notch in the first checkpoint. They found that B cells overexpressing Dll1 induce the development of well differentiated cortical an medullary microenvironments in dGuo-treated FTOC lobes, proposing a model in which TECs and thymocytes support each other through bi-directional Notch signaling. Nevertheless, they do not provide solid evidence of Dll1-induced Notch signaling activation on TECs. Conversely, our data provide the first *in vivo* evidence of Notch signaling activation in murine, and more importantly, human TECs. Nevertheless, further study of Notch signaling activation in human fetal TECs is compulsory to confirm the role of Notch in the early steps of human TEC development. However, previous data showing Notch1 activation in murine fetal TECs support the involvement of Notch1 in TEC ontogeny (Del Monte et al., 2007). Additionally, the strong expression of the canonical Notch target gene Hes1 in postnatal TECs, as well as the specific activation of Notch1 in mTECs and scTECs, but not in cTECs, suggest that Notch signaling activation is mediated by different Notch receptors in cTECs and mTECs. This is not a surprising finding as scTECs are developmentally related and phylogenetically more similar to mTECs than to cTECs (McFarland et al., 1984) (Haynes et al., 1984). This would also explain our previous findings regarding the specific upregulation of Jag1 and Jag2 by subcapsular cTECs in the involuted thymus, meaning that with thymic involution scTECs acquire the phenotypic characteristics of their medullary counterparts. Evidences of the involvement of Notch signaling in the second phase of TEC development are equally scarce, as only one report suggested the involvement of Notch signaling in postnatal TECs homeostasis and thymic involution (Beverly et al., 2006). Nevertheless, we found that Notch signaling activation in mTECs increases with age, supporting the role for Notch in thymic involution. A kinetic study in postnatal murine thymus allowed us to identify the precise time-point on which Notch1 activation significantly increases in mTECs, corresponding with the age of 3 months-old. As human thymus developmental kinetics are faster than murine, in

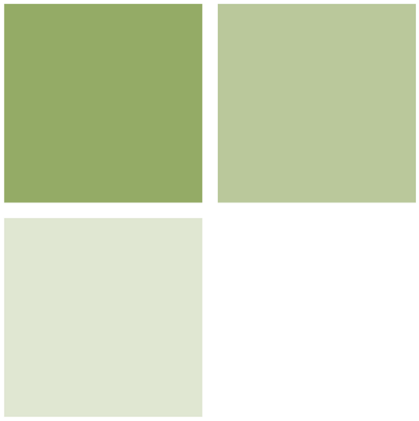
humans this point might correspond with the neonatal thymus or even the late stages of fetal thymic development, thus human fetal ICN1 staining are again necessary to define at which point Notch1 signaling is upregulated in TECs.

In order to specifically dissect the role of Notch in TEC development, we established a conditional mouse model in which Notch signaling is specifically abrogated in epithelial cells. The resulting RBPjκ-KO^{TEC} mice were phenotypically normal during their first 7 months of age, but aged mice presented defects in hair growth and developed skin lesions as previously described (Demehri et al., 2008) (Demehri et al., 2009) (Dumortier et al., 2010). Histological analysis of the lesions showed a granulomatous-like skin disease. In the thymus, Hes1 staining provided evidence of specific Notch signaling abrogation in RBPjk-KO TECs, although we observed differences in Hes1 expression levels between different KO mice (data not shown), implying that deletion efficiency may vary from different individuals. Anyhow, *in vivo* immunohistochemical Hes1 staining provided a powerful tool to assess Notch signaling activation *in vivo*. A solid implication of Notch in TEC development was provided by cortico-medulary compartmentalization analyses of RBPjk-KO thymi, which showed a clear defect in the medullary microenvironment size; result double-checked by FACS analysis of cTEC and mTEC populations (data not shown). The choose of a medullary marker for this analyses was not trivial, specially when studying medullary size in old age animals where thymic involution may have already disorganized the thymic microenvironment and deregulated the expression of several epithelial markers such as cytokeratins, MHC and more importantly Notch ligands expression (Ortman et al., 2002) (Aw et al., 2008) (Aw et al., 2009) (Fiorini et al., 2008). However, we found no differences in Notch ligand expression in mTEC in the involuted thymus except for Dll1, which was upregulated in terminally differentiated mTECs including HCs. Nevertheless, we have to consider that Notch signaling regulates the expression of the elements of its own pathway, as for example *NOTCH1* and *NOTCH3* genes (Ikawa et al., 2006) (Del Monte et al., 2007) (Van de Walle et al., 2009), thus when abrogating Notch signaling in our conditional RBPjk-KO^{TEC} mice, we could be disrupting the Notch signaling positive feedback loop that may also regulate Notch ligands expression in TECs. Indeed, this fact would explain the observed ectopic appearance of B cells in the thymus. Indeed, it has been demonstrated that Notch signaling regulates Dll1 and Jag2 expression; being the later a direct target of c-myc (Yustein et al., 2010) (Fiaschetti et al., 2014), which in turn is a well-known target of Notch1 (Palomero et al., 2006). In this line of arguments we could not exclude the possibility that the observed reduction of Jag1+ stained area in RBPjk-KO^{TEC} thymi could be due not to a reduction in medulla size, understood as less number of mTECs, but to a reduced expression levels of Jag1. However, this scenario is unlikely since: (1) FACS analysis of TEC populations in WT and RBPjk-

KO^{TEC} mice corroborate the histomorphometric results as RBPjk-KO^{TEC} mice showed less frequency (%) of mTECs than WT mice, (2) there are no evidences in the literature reporting Jag1 as target of the Notch signaling pathway; (3) independently to a putative regulation of Jag1 expression by Notch signaling, Jag1 is expressed in medullary-exclusive non-epithelial cells, as 30% of CD11c+ myeloid cells and some perivascular cells express Jag1, both cell types *per se* defining the medullary microenvironment; (4) the medulla is not only defined by medullary specific markers but also by its differential histology with the cortex. Even without a medullary marker, the thymic medulla can be defined attending to low cellular density observed by Topro3 staining; (5) regular Jag1+ staining in mTECs was observed in every remaining medullary areas of the RBPjk-KO^{TEC} thymi, data altogether confirming that Jag1 is a good thymic medullary marker. Nevertheless, numerous questions remains unanswered regarding the role of Notch signaling in thymic compartimentalization and medulla formation. Two possibilities may account medulla size reduction in RBPjk-KO mice: (1) Notch regulates the senescence process inducing mTEC apoptosis in the involuted thymus, as suggested by Capobianco et al. (Beverly et al., 2006). (2) There is a defect in mTEC fate specification and thus medulla formation during development, which would be supported by the fact that the medullary patches observed in our RBPjk-KO mice mimic the clonal mTEC islets from which medulla derives during thymus ontogeny (Rodewald et al., 2001).

An interesting observation derived from our *in vivo* immunohistological studies in mice and human thymus is that highest Notch signaling activation (Hes1 or ICN1) corresponded with mTECs forming the HCs. The role of the HCs in thymus biology is still a matter of debate. Traditionally, HCs has been considered senescent structures formed by terminally differentiated mTECs. But mTECs forming HCs are connected to one another by desmosomes and exhibit long cytoplasmic processes that directly contact thymic T lymphocytes and accessory antigen presenting cells (Bodey and Kaiser, 1997). mTECs forming the outer layer of Hassal's bodies, as well as scTECs and terminally differentiated mTECs, are very similar to the basal layer of epidermis, as they exhibit close antigenic similarity with skin epithelial cells (Kampinga et al., 1989) and all the classical characteristics of an epithelium: they are polarized, form hemidesmosomes and highly express cytokeratins (Petrie, 2002), thus resembling to a finally differentiated epithelial cell similar to the stratum corneum keratinocytes (Haynes, 1984) (Schmitt et al., 1987), which, by the way, efficiently supports T cell development (Clark et al., 2005). It has been shown that p53 is active in scTECs and HC mTECs of the normal human thymus (Jablonska-Mestanova et al., 2013), a protein well known by its anti-apoptotic and tumor suppression function in the skin. Further inmunohistochemical studies of p53 and other pro-apoptotic proteins such as Bcl2 and survivin in RBPjk-KO^{TEC} thymi, as well as in the human thymus, would give additional

valuable hits about the putative role of Notch in TEC senescence and homeostasis. Additionally, RBPjk-KO^{TEC} thymi hosted a great proportion of B cells and showed a significant reduction in the frequency of DP thymocytes. As suggested earlier, B cell appearance in this KO thyme might be due to Notch ligand expression downregulation, and an abnormal DP thymocyte compartment could be the cause of the defective medulla size, as DP thymocytes are needed for terminal mTEC differentiation. It is interesting to observe that RBPjκ-KO^{TEC} mice show an increased proportion in DP B220+ Thy1+ thymocytes, being the B220 marker associated in T cells with the apoptotic process (Renno et al., 1998), thus suggesting that DP thymocytes are subjected to higher rates of apoptosis in RBPjk-KO^{TEC} than in WT mice; annexinV expression analysis would shed light into this issue. Nevertheless, as sampling population is mainly composed by aged mice, we cannot exclude that the DP defective population may be attributable to age-related stress or to the systemic illness derived from Notch signaling abrogation in the skin and other epithelial organs. It has been shown that Notch signaling abrogation in the skin cause a systemic inflammatory and lymphoproliferative disease mostly driven by the cytokine TSLP secreted by skin keratinocytes (Demehri et al., 2008) (Dumortier et al., 2010). TSLP secretion acts as a tumor suppressor in the skin somehow compensating the loss of Notch (Di Piazza et al., 2012), which has been described to be tumorigenic (Demehri et al., 2009). Interestingly, one of the main and well-established functions of the HCs, is TSLP secretion. Thus, we cannot exclude the possibility that the observed defect in the DP compartment and B cell appearance in the thymus might be the consequence of increased TSLP secretion in the within the thymus from Notch-deficient HC mTECs, as it has been demonstrated that TSLP induces human B cell proliferation and differentiation (Scheeren et al., 2010). Interestingly, the molecular structure of TSLP is very similar to IL7, a cytokine essential for T cell development, as both cytokines binds the IL-7Rα chain. Indeed, the phenotype found in our RBPjk-KO^{TEC} mice is very similar to that observed in IL7-Tg mice (Rich et al., 1993), which show marked reduction of DP thymocytes and also develop a progressive cutaneous disorder involving dermal lymphoid infiltrates resulting in progressive alopecia, hyperkeratosis, and exfoliation. Additionally, IL7 transgene also provokes the development of a lymphoproliferative disorder that induces B and T cell lymphomas. In summary, our data confirms that the relevance of Notch signaling in the thymus can be extended from the T cell compartment to the main thymic stromal compartment, the TECs, as Notch signaling abrogation modifies thymic architecture and thymocyte populations, by a mechanisms still unknown but that may involve defective regulation of apoptosis and secretion of TSLP by mTECs. **Thus a Notch signaling crosstalk may exists between TECs and thymocytes, or between TECs themselves, as reciprocal Notch signaling interactions are required to achieve complete maturation of both cell types as well as homeostasis of the whole thymus during postnatal life.**



Conclusions

1. Notch ligands and receptors are differentially expressed throughout the thymus, thus defining specific niches that vary during thymus ontogeny and age-related thymic involution, and differ from human to mice.

2. Dll4, the essential Notch ligand for T cell development, is early downregulated from cortical TECs during human thymus ontogeny.

3. Vascular endothelium as well as perivascular cells and myeloid cells at the CMJ provide an important source of Dll4 ligands to progenitors entering the human thymus, suggesting that Dll4 may contribute to Notch activation at very early T cell developmental stages.

4. Notch1 is active *in vivo* in human CD34+ progenitors located at the CMJ and in a minute population of CD34- cortical thymocytes, indicating that Notch1 signalling is transiently induced early in human T cell development.

5. Jag1 and Jag2 Notch ligands define reciprocal medullar and cortical niches, respectively, in the human thymus and exhibit opposite roles in the generation of αβ versus γδ T cells, but they do not seem to impact the transcriptional priming of the γδ T cell lineage, at least before acquisition of the TCRγδ.

6. Jag2 signaling impairs the generation of αβ-lineage cells at the β-selection check-point, but efficiently supports γδ T cell development and selectively promotes the expansion of γδ T cells at the CD1a+ immature CD4+ and DP developmental stages.

7. Jag1 is the most efficient Notch ligand at supporting the developmental progression of CD1a+ γδ T cells and the generation of mature CD1a- DN and CD8+ γδ T cells with functional potential.

8. Two distinct human thymus niches, the CMJ and the medulla, host γδ thymocytes with different expression levels of the CXCR4 chemokine receptor, which are placed at sequential CD1a+ and CD1a-/lo γδ T cell developmental stages. Therefore, final maturation of cortical CD1a+ γδ thymocytes may involve their migration into the thymus medulla.

9. Notch1 signaling is active in cortical and medullary TECs, and selectively increases with age in the latter, in both mouse and human, suggesting a role for Notch1 in medullary TEC homeostasis and age-related thymus involution.

10. Selective abrogation of Notch signaling in mouse TECs *in vivo*, disturbs the architecture of the thymus medulla and impacts thymus function by impairing DP thymocyte development while inducing the aberrant generation of intrathymic B cells.

Conclusiones

1. Los ligandos y receptores de Notch se expresan diferencialmente en el timo, definiendo nichos específicos que varían durante su ontogenia e involución asociada a la edad, y que difieren en humanos y ratón.

2. Dll4, el ligando de Notch1 esencial para el desarrollo de los linfocitos T, disminuye sus niveles de expresión en las TEC corticales en estadios tempranos de la ontogenia tímica humana.

3. El endotelio vascular, así como las células perivasculares y las células mieloides de la CMJ, proporcionan una fuente importante de ligando Dll4 a los progenitores que colonizan el timo humano, lo que sugiere que Dll4 puede contribuir a la activación de Notch en etapas muy tempranas del desarrollo T.

4. Notch1 se activa in vivo en progenitores intratímicos tempranos CD34+ situados en la CMJ y también en una mínima población de timocitos corticales CD34-, lo que indica que la señalización por Notch1 se induce tempranamente y de forma transitoria durante el desarrollo de las células T humanas.

5. Los ligandos de Notch Jag1 y Jag2 definen nichos recíprocos, medular y cortical, respectivamente, en el timo humano, y desempeñan funciones opuestas en la generación de células T de los linajes αβ versus γδ, pero no parecen afectar el programa transcripcional específico del linaje T γδ , al menos antes de la adquisición del TCRγδ.

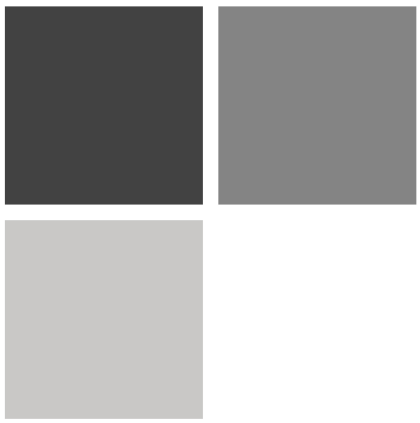
6. La señalización mediada por Jag2 afecta negativamente a la generación de células del linaje αβ en el punto de control de la selección β, pero favorece el desarrollo de las células T γδ y promueve selectivamente la expansión de los timocitos γδ en estadios immaduros CD1a+ CD4 + y CD1a+ DP del desarrollo intratímico..

7. Jag1 es el ligando de Notch más eficiente en inducir la progresión de las células γδ immaduras CD1a+ y la generación de células T γδ CD1a- DN y CD8+ funcionalmente maduras.

8. La unión corticomedular y la médula, definen dos nichos en el timo humano que albergan timocitos γδ con diferentes niveles de expresión del receptor de quimioquinas CXCR4 y de CD1a, siendo los timocitos γδ corticales los que expresan mayores niveles de ambos marcadores, por lo que la maduración final de los timocitos γδ corticales inmaduros CD1a+ implicaría su migración a la médula del timo.

9. Notch1 señaliza en TECs corticales y medulares y su activación aumenta selectivamente en estas últimas con la edad, tanto en humanos como en ratón, lo que sugiere la implicación de Notch1 en la homeostasis de las TEC medulares y en la involución del timo relacionada con la edad.

10. El bloqueo selectivo de la señalización por Notch en las TECs de ratones condicionales deficientes perturba la arquitectura del nicho medular, y altera la función tímica, impidiendo la generación de timocitos DP e induciendo la generación aberrante de células B intratímicas.



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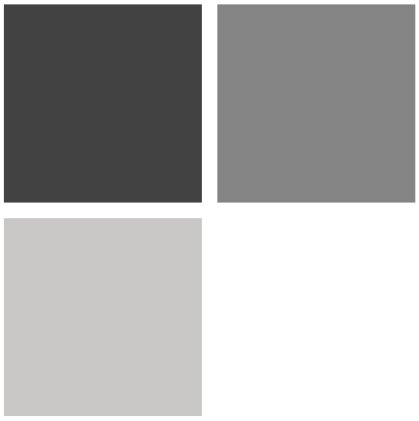

Supplementary Information

Supplementary Figures:

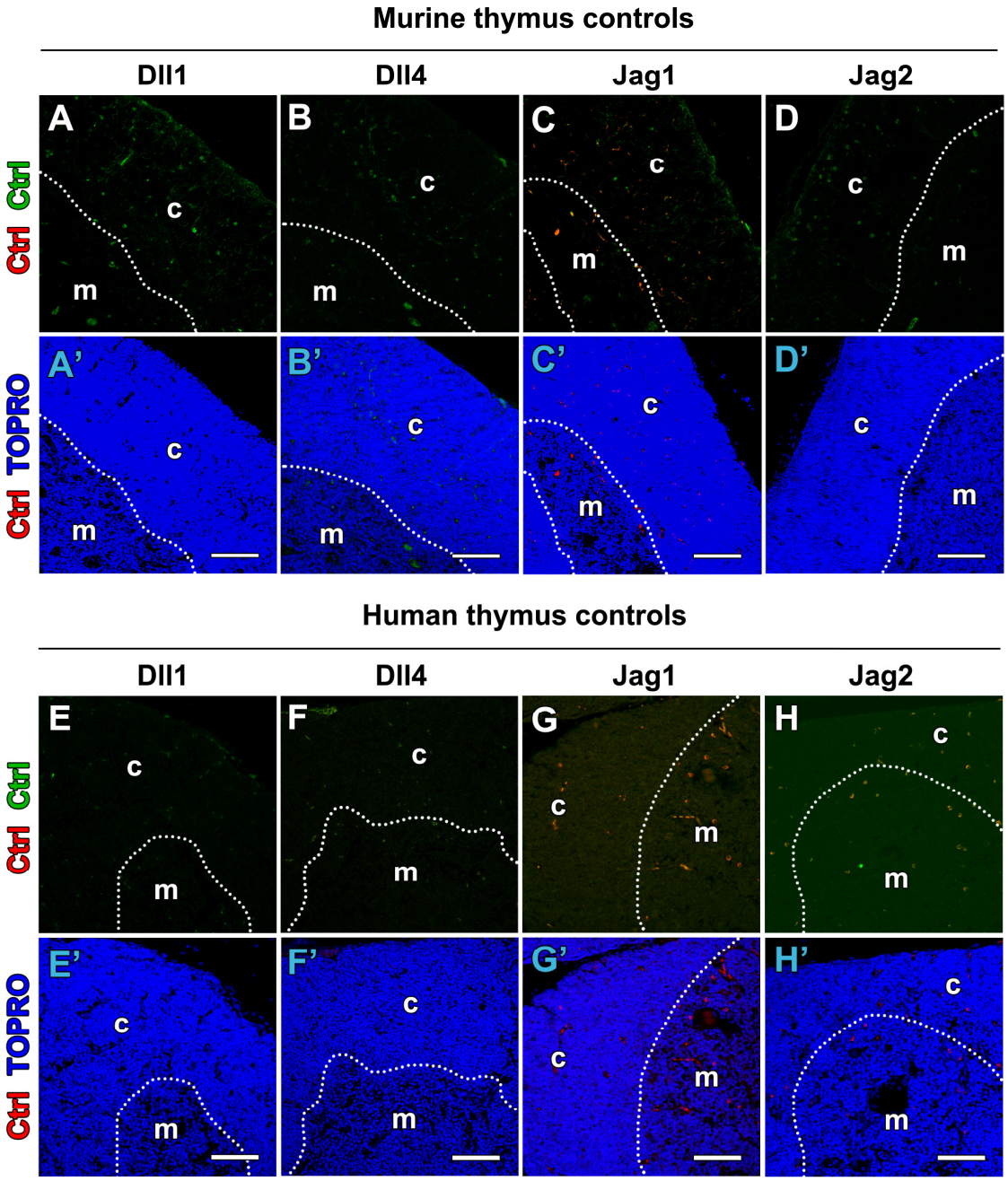

Figure S1. Notch ligands and panCK control staining in the murine and human thymus. Images show FFPE sections of murine postnatal (< 16 week-old; *upper panel*) and human postnatal thymic tissue (< 17 months-old; *lower panel)* stained in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). Thymic regions are labeled as *c:* cortex; *m:* medulla; *dotted line:* CMJ. Scale bar: 100µm. Images shown are representative of results obtained in $N \geq 3$ tissue samples per ligand.

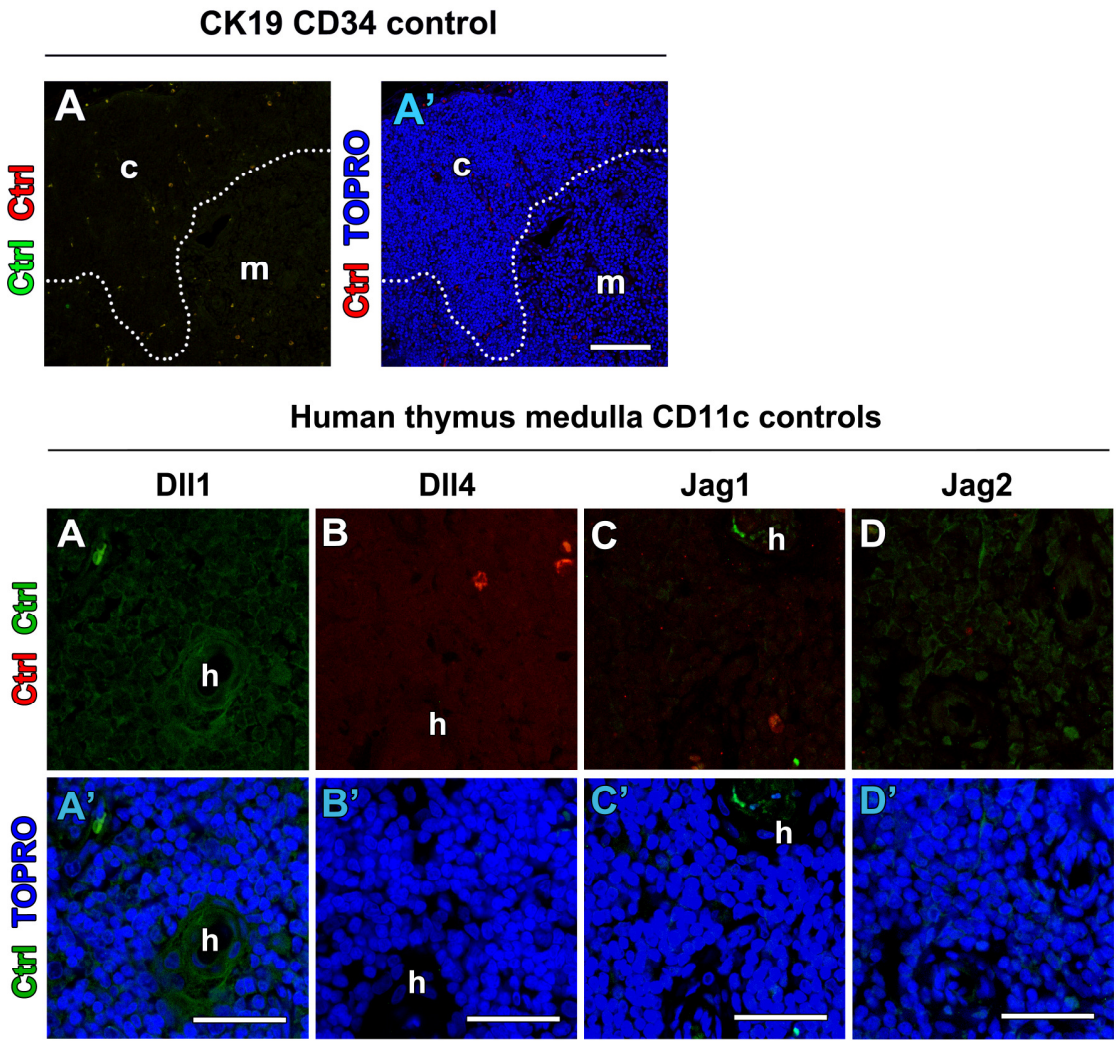

Figure S2. Notch ligands and stromal markers CK19, CD11c and CD34 control staining in the human thymus. Images show FFPE sections of human postnatal thymic tissue (< 17 months-old) stained in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). Thymic regions are labeled as *c:* cortex; *m:* medulla; *dotted line:* CMJ. Upper panel scale bar: 100µm. Lower panel scale bar: 50µm; Images shown are representative of results obtained in $N \geq 3$ tissue samples per ligand.

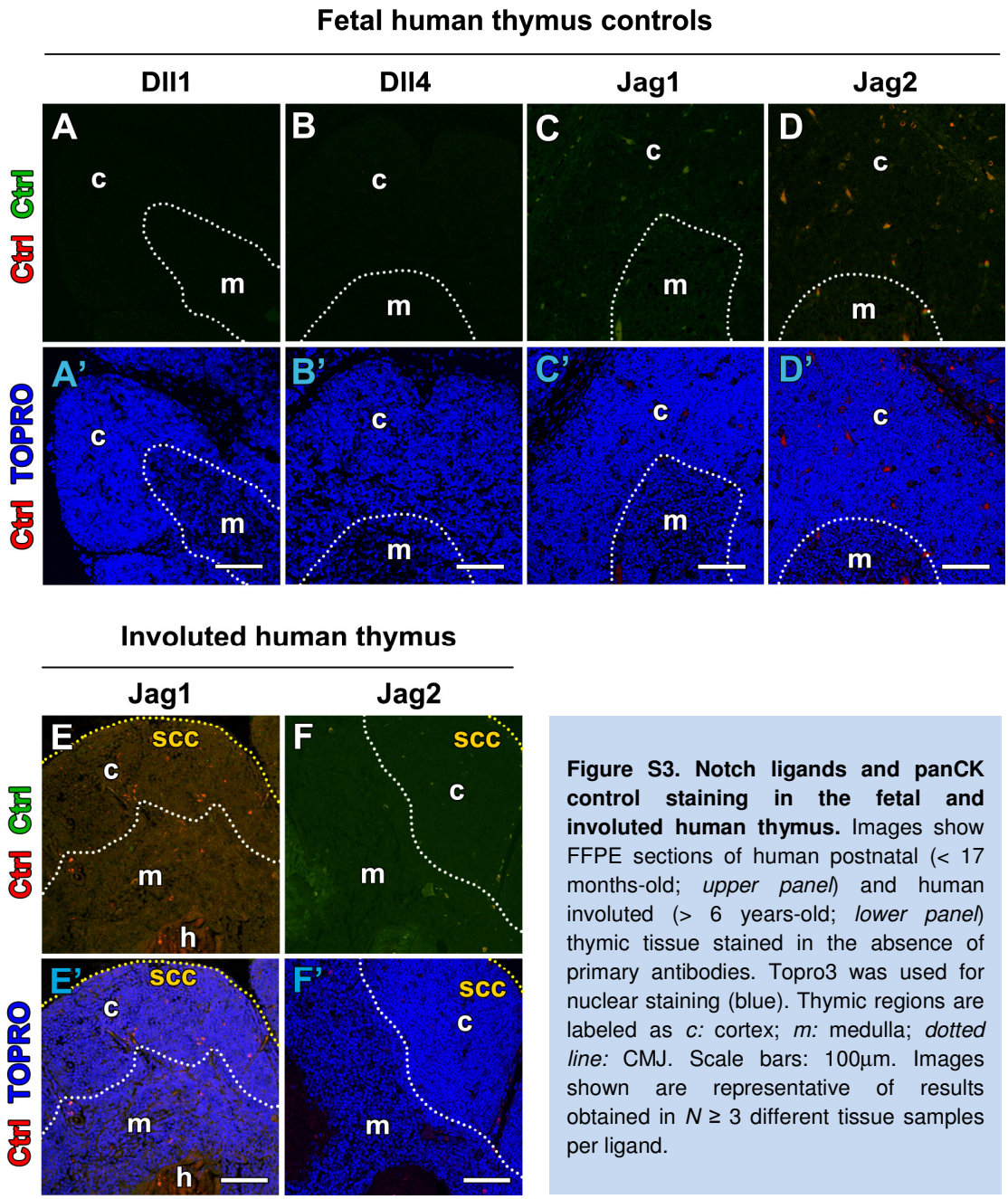

Figure S3. Notch ligands and panCK control staining in the fetal and involuted human thymus. Images show FFPE sections of human postnatal (< 17 months-old; *upper panel*) and human involuted (> 6 years-old; *lower panel*) thymic tissue stained in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). Thymic regions are labeled as *c:* cortex; *m:* medulla; *dotted line:* CMJ. Scale bars: 100µm. Images shown are representative of results obtained in $N \geq 3$ different tissue samples per ligand.

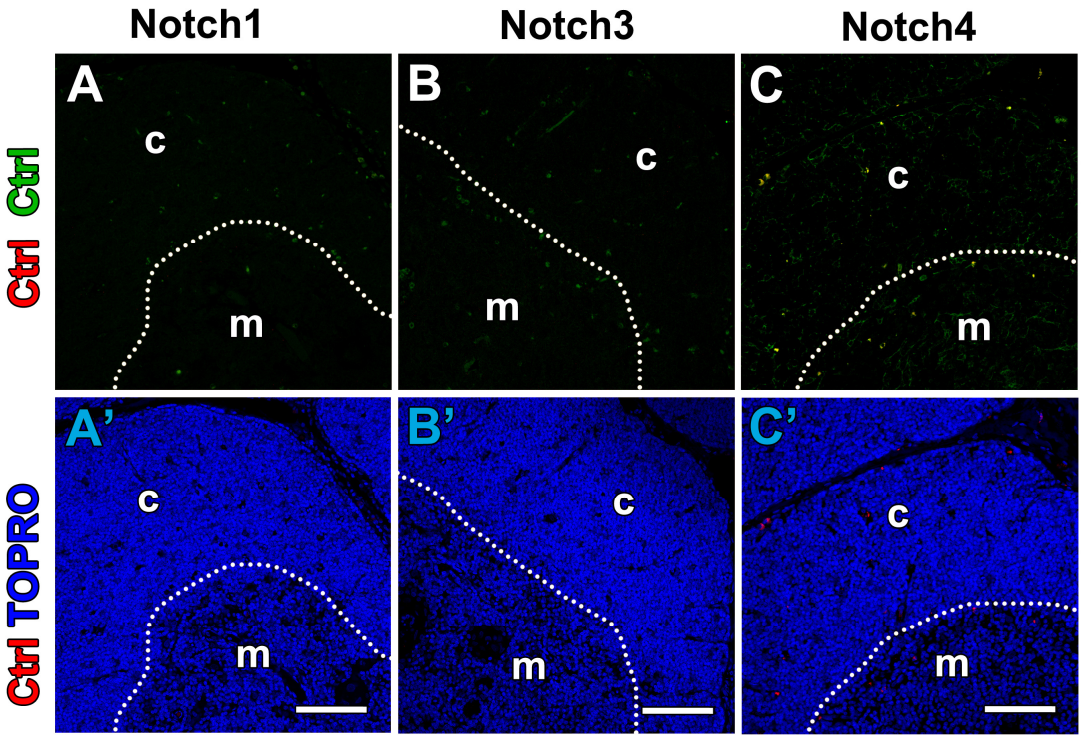

Figure S4. Notch receptors and panCK control staining in the human thymus. Images show FFPE sections of human postnatal (< 17 months-old) thymic tissue stained in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). Thymic regions are labeled as *c:* cortex; *m:* medulla; *dotted line:* CMJ. Scale bars: 50µm; Images shown are representative of results obtained in $N \geq 3$ different tissue samples per receptor.

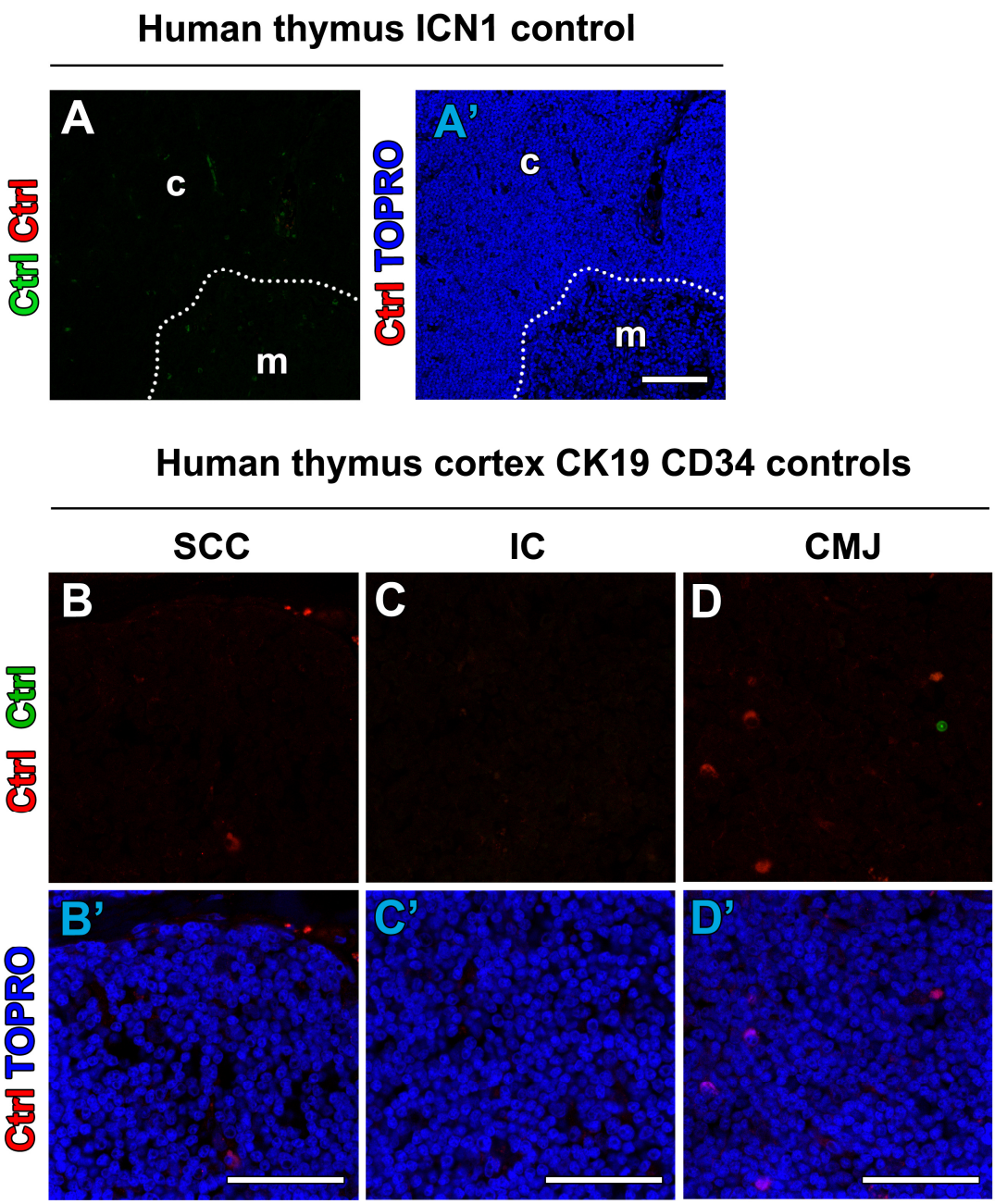

Figure S5. Intracelullar Notch1 and cortical CD34 and CK19 control staining in the human thymus. Images show FFPE sections of human postnatal (< 17 months-old) thymic tissue stained in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). Thymic regions are labeled as *c:* cortex; *m:* medulla; *dotted line:* CMJ. Upper panel scale bar: 100µm. Lower panel scale bars: 50µm; Images shown are representative of results obtained in $N \geq 3$ different tissue samples per ligand.

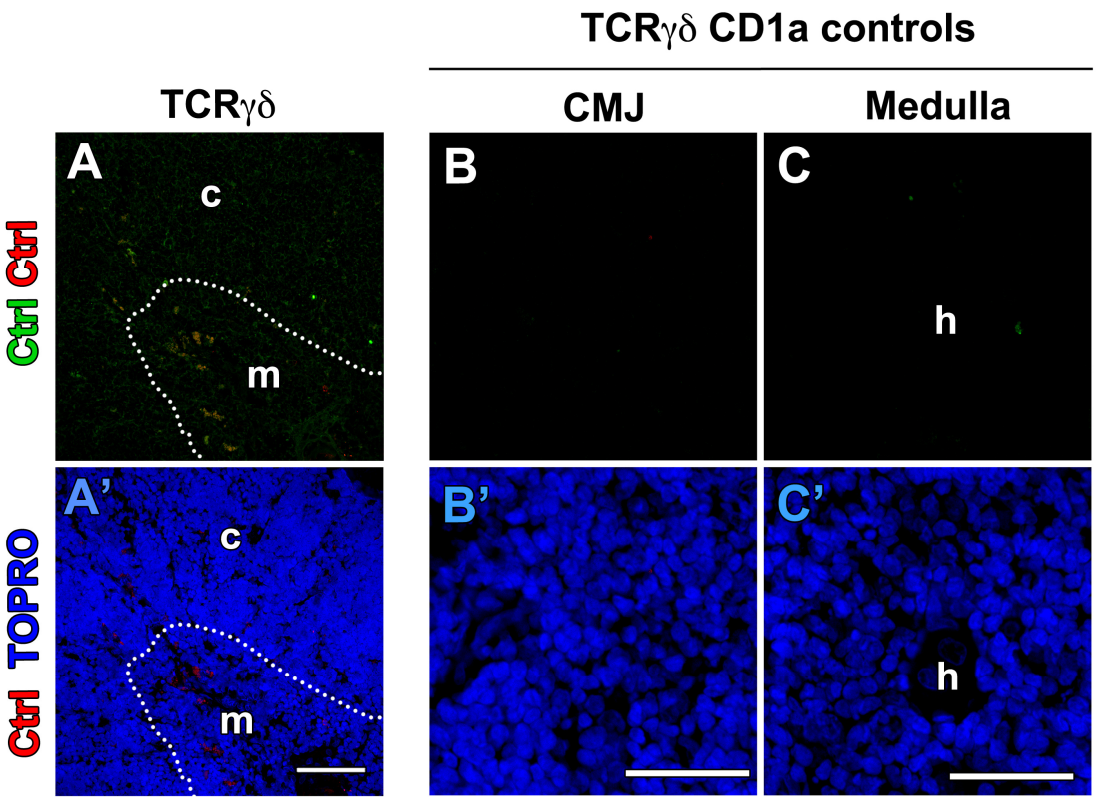

Figure S6. TCRγδ and CK19 control staining in the human thymus. Images show snap-frozen sections of human postnatal (< 17 months-old) thymic tissue in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). Thymic regions are labeled as *c:* cortex; *m:* medulla; *dotted line:* CMJ. Left panel scale bar: 100µm. Right panel sacale bar: 50µm; Images shown are representative of results obtained in $N \geq 3$ tissue samples per ligand.

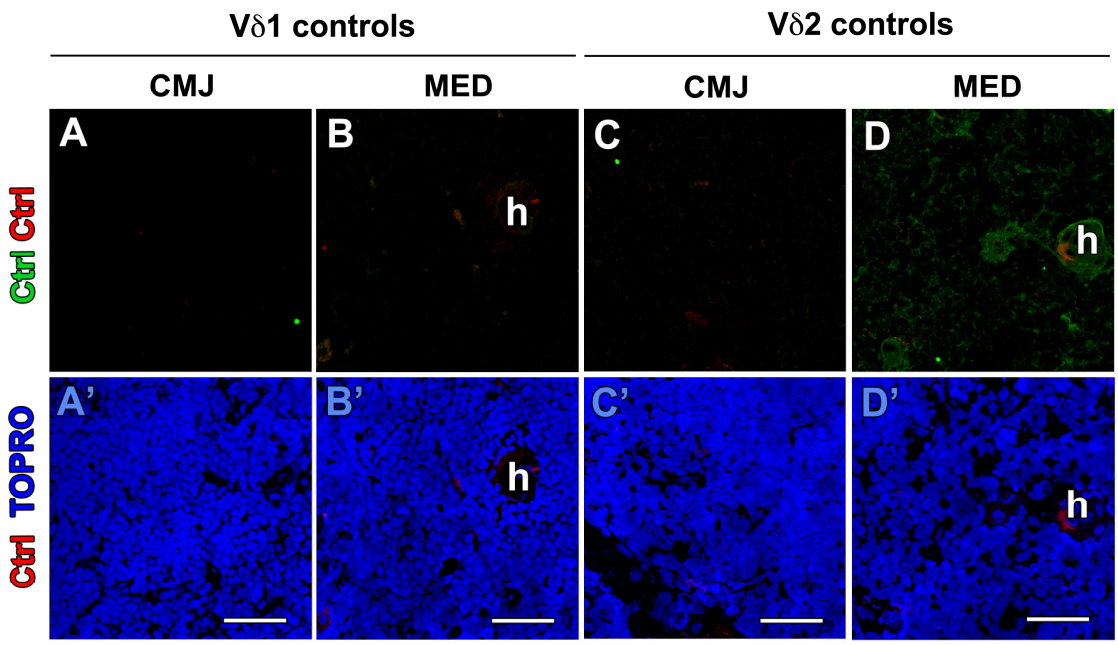

Figure S7. Vδ1, Vδ2 and CK19 control staining in the corticomedulary and medullary microenvironments of the human thymus. Images show snap-frozen sections of human postnatal (< 17 months-old) thymic tissue stained in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). *h:* Hassal's corpuscle. Scale bars: 50µm; Images shown are representative of results obtained in $N \geq 3$ different tissue samples per ligand.

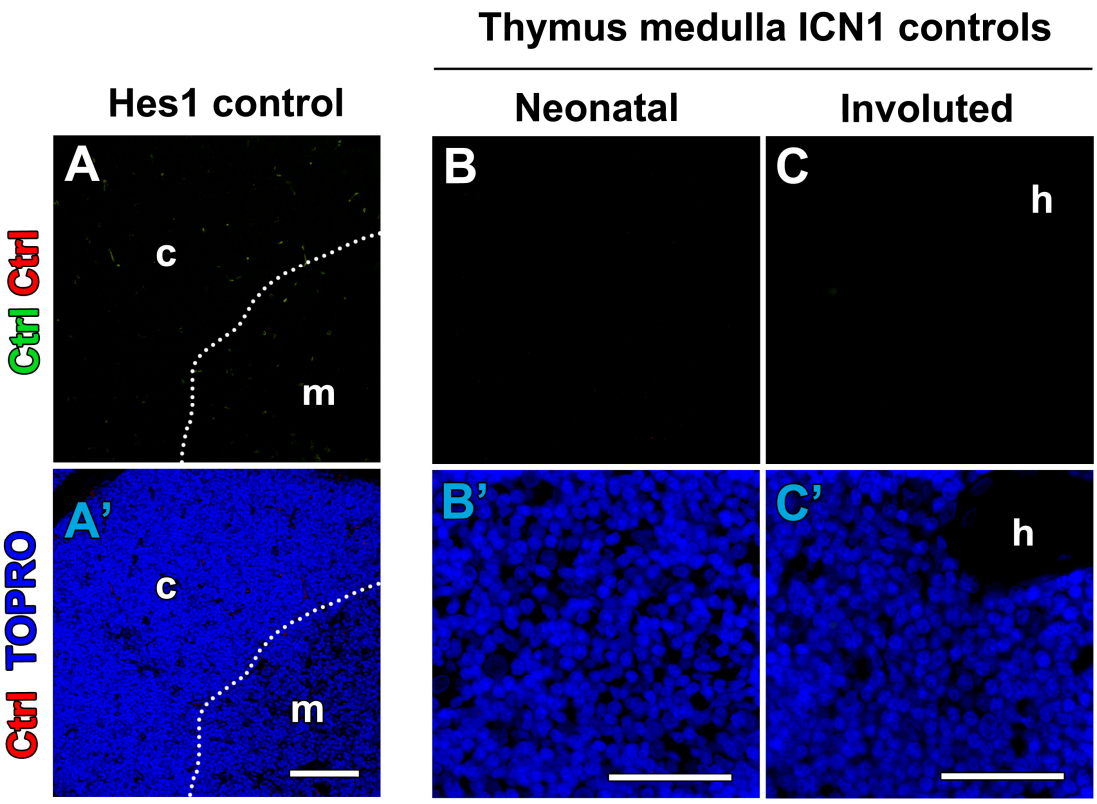

Figure S8. Hes1 and medulary intracellular Notch1 and panCK control staining in the human thymus. Images show FFPE sections of human postnatal (< 17 months-old) thymic tissue stained in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). Thymic regions are labeled as *c:* cortex; *m:* medulla; *dotted line:* CMJ. Left panel scale bar: 100µm. Right panel scale bars: 50µm; Images shown are representative of results obtained in $N \geq 3$ different tissue samples per ligand.

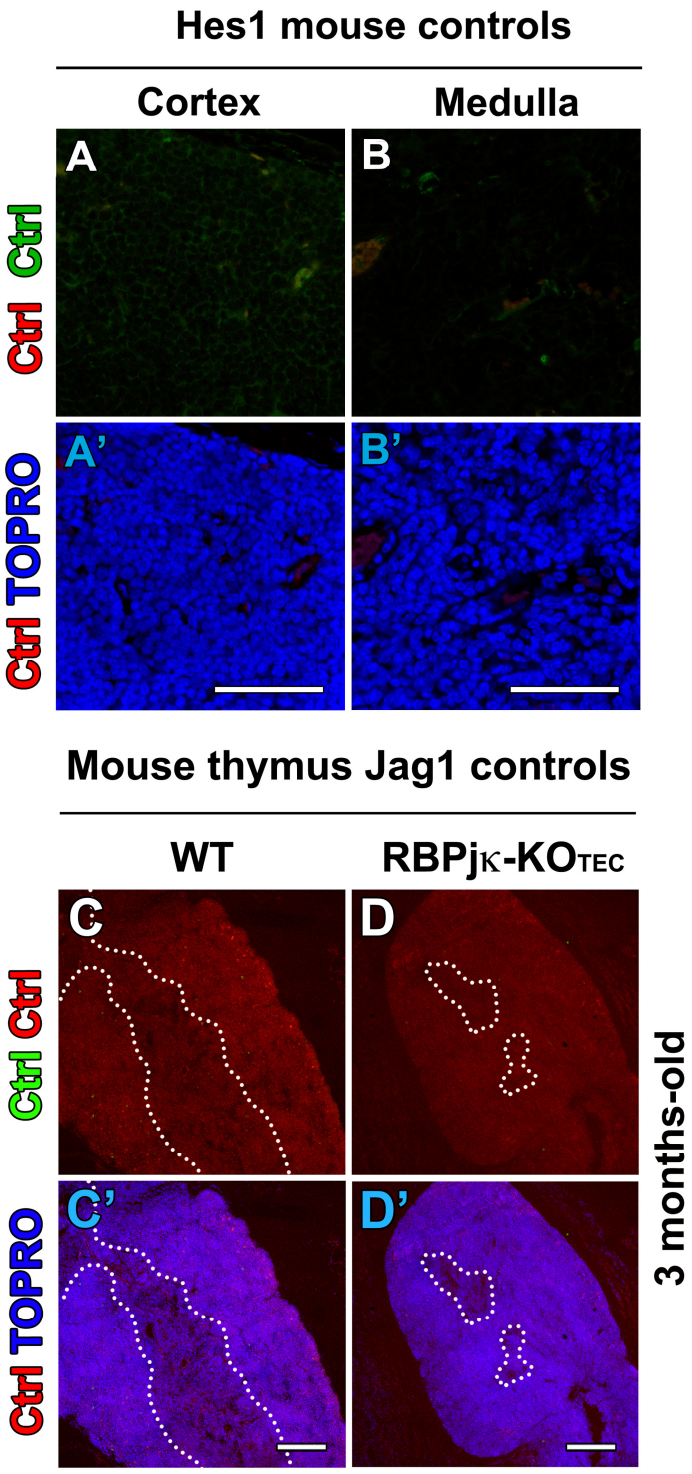

Figure S9. Hes1, Jag1 and panCK control staining in WT and RBPjk-KO^{TEC} mice thymi. Images show FFPE sections of murine postnatal (3 months-old) thymic tissue stained in the absence of primary antibodies. Topro3 was used for nuclear staining (blue). *Dotted line:* CMJ. Upper panel scale bar: 50µm. Lower panel scale bars: 200µm; Images shown are representative of results obtained in *N* ≥ 7 different tissue samples.